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TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY
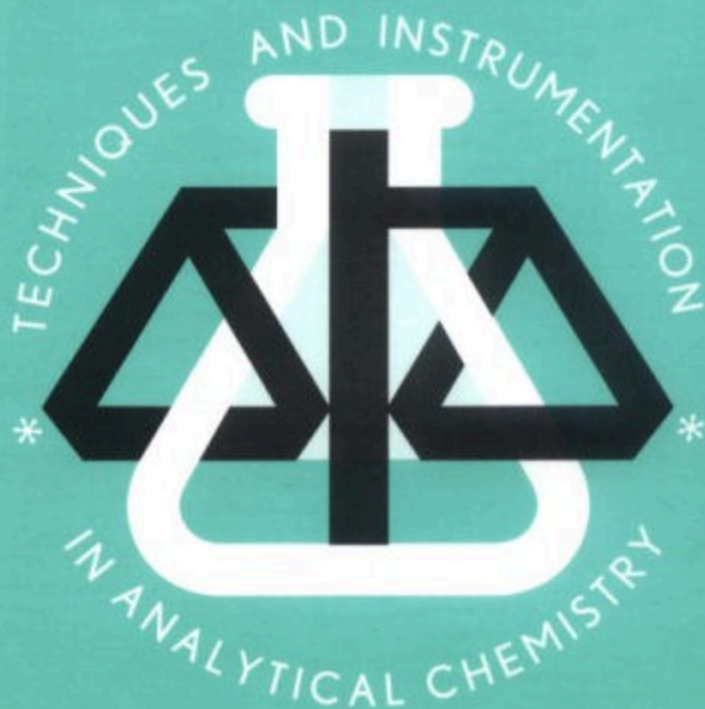
SAMPLE HANDLING AND TRACE ANALYSIS OF POLLUTANTS
TECHNIQUES, APPLICATIONS AND QUALITY ASSURANCE

edited by

D. BARCELÓ

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PREFACE

This book is an updated, completely revised version of a previous volume in this series entitled: *Environmental Analysis – Techniques, Applications and Quality Assurance*. The book treats different aspects of environmental analysis such as sample handling and analytical techniques, the applications to trace analysis of pollutants (mainly organic compounds), and quality assurance aspects, including the use of certified reference materials for the quality control of the whole analytical process. Besides updating the previous book, new analytical techniques are presented that have been developed significantly over the last 6 years, like solid phase microextraction, microwave-assisted extraction, liquid chromatography-mass spectrometric methods, immunoassays, and biosensors. Not all the authors of the previous version were able to update their chapters, three of them because there had been changes in their fields of interest. However, new authors have been incorporated and the book has grown from 17 chapters to 22 chapters.

The book is divided into four sections. The first describes field sampling techniques and sample preparation in environmental matrices: water, soil, sediment and biota. It provides a critical review of different sample handling strategies in the analysis of organic pollutants in the aquatic environment, with emphasis on a variety of techniques like solid phase extraction and solid phase microextraction for water analysis, microwave-assisted extraction for soil and sediment samples, off-line and on-line strategies for water analysis and a variety of clean-up methods for isolating persistent pollutants from sediment and biota samples.

The second section covers the application areas and contains the largest number of chapters. Applications are either based on techniques, like the use of gas chromatography-atomic emission detection, immunoassays, or coupled-column liquid chromatography, or on specific application areas, like chlorinated compounds, pesticides, phenols, mycotoxins, phycotoxins, radionuclides, industrial effluents and wastes, including mine waste. This section is particularly relevant since it shows the performance of analytical techniques for the determination of trace pollutants in real-world environmental samples.

Validation and quality assurance are key parameters in all measurements. These aspects are described in two chapters dealing with the use and preparation of reference materials that will guarantee the quality control of the whole analytical process. A third chapter in this section covers the interpretation of environmental data using advanced chemometric techniques that will guarantee a better interpretation and quality of the data reported.

The final section, entitled Emerging Techniques, reports the use of somewhat advanced analytical methods, usually more expensive, less routinely used or less developed, for the determination of pollutants. In this section the different forms of capillary electrophoresis are reported together with the latest development in liquid chromatography-mass spectrometry and mass spectrometric methods in general. The use of different hyphenated tech-

niques for speciation and analysis and the application of biosensors in environmental analysis are also included.

The book is intended to serve both as general reference for postgraduate students as well as a practical reference for environmental chemists who need to use analytical techniques for environmental studies and analytical chemists needing information on the complexity of environmental sample matrices and interferences. Each chapter includes sufficient references to the literature to serve as a valuable starting point for a more detailed investigation. By comparing this book with its predecessor, the reader can trace the tremendous developments achieved during the last decade in this particular field of analytical chemistry .

Finally I would like to thank the authors for their time and effort in preparing their chapters. Without their cooperation and engagement this volume would certainly not have been possible.

D. Barceló

Chapter 1

Sample handling strategies for the analysis of organic compounds in environmental water samples

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1.1 INTRODUCTION

In the former edition of this book, this chapter began by pointing out the increasing need for monitoring greater numbers of hazardous organic substances at lower and lower levels due to new rules and regulation being set up by many countries for environmental protection. During the last 6 years, in the European Union (EU), several directives gave the priority to surface water quality and to the control of organic pollutants in industrial effluents discharges and wastewater. Therefore, today it is necessary to add that more and more complex matrices have now to be monitored.

Organic compounds present in environmental waters may be naturally occurring compounds, anthropogenic compounds or degradation products from industrial and urban rejects and agricultural activity. For example, traces of pesticides and their transformation products are regularly detected in ground and surface waters. The occurrence of organic compounds in surface water is still in trace amounts at the microgram per litre (pbb) levels and below for most of contaminants. They can have very different polarities and chemical properties. In EU, the drinking water ordinance sets a limit in concentration 0.1 µg/l for each pesticide, so that detection limits below the 0.1 µg/l level are required for monitoring drinking water. Such low detection limits are also necessary for studying the fate and the transport of organic compounds directly in environmental waters. Screening for low levels of this large variety of compounds requires high performance from analytical instruments as well as sample preparation techniques.

Determination of organic compounds is generally carried out by gas (GC) or liquid (LC) chromatography, depending on their polarity, volatility and the risk of decomposition at high temperature. In general, environmental water samples cannot be analysed without some preliminary sample preparation because they are too dilute and too complex. Preconcentration of samples of relatively large volume is necessary to overcome the limitation of the detection system, but the extract is often too complex for an efficient separation by the chromatographic column at low detection levels. Sample pretreatment is therefore an essential part of the whole chromatographic procedure. Its objective is to provide a sample fraction enriched in all the analytes of interest, and as free as possible from other matrix components. This pretreatment, which can be achieved in one to three different steps, consists in (i) extracting traces of analytes of interest from the aqueous media, (ii) concentrating these traces, (iii) removing from the matrix other components which have been co-extracted and co-concentrated and which may interfere in the chromatographic analysis (i.e. clean-up).

Before implementing any strategy, it is important to consider the strong interdepen-

dence of the various steps of the whole analytical procedure:i.e. the sample handling, separation and detection. There is no unique strategy for the sample pretreatment of organic compounds in waters. It mainly depends on the nature of the solutes to be determined (e.g. volatility, polarity, molecular weight), on the nature of the matrix and on the level of concentration required. Interference removal is a critical step which depends strongly on the concentrations of analytes of interest and of the nature of the aqueous media. In other words, the strategy for determining a pesticide below the microgram per litre level in drinking water will be different from that used for very polluted river water. It will also be guided by the separation, and especially by the method of detection mode. If a very selective detection can be carried out, the sample handling may be simplified, whereas a simple detection mode can be used if a selective detection mode is applied. This 'total system' approach is of prime importance for selecting the optimal sample handling strategy [1].

The sample pretreatment is still the weakest link and the time-determining step in the whole analytical procedure and the primary source of errors and discrepancies between laboratories. Volatile organic compounds are analysed by gas chromatography (GC) techniques and their sample pretreatment is carried out using specific techniques, which are relatively easy. In contrast, the sample handling of non-volatile organic compounds is more difficult, especially because of the numerous other non-volatile analytes of the matrix. Therefore, highly selective sample pretreatment sometimes requires sophisticated methods, especially if a detection limit of a few nanograms per litre level is required in a complex matrices where interferents are at higher amounts.

The aims in the determination of organic compounds in environmental water samples can be to give a broad-spectrum analysis, with determination and identification of the largest possible number of known and unknown analytes at one time, or the determination of one or several target compounds. The first approach requires a non-selective preconcentration, and is straightforward, but the extract is often complex and has to be fractionated before analysis. In the second approach, carrying out a selective preconcentration of target analytes is more challenging, and always more rapid.

Trace-enrichment can be performed by liquid–liquid or liquid–solid extraction techniques. Liquid–liquid extraction (LLE) has remained the preferred technique for several years, but today, solid-phase extraction (SPE) is fully accepted as the alternative sample preparation method to LLE in many official methods by regulatory agencies in North America and Europe [2–13]. A first reason is that SPE has now become a reliable and useful tool for sample handling, with an increasing choice of available solid sorbents. A second one was the pressure the decrease organic solvent usage in laboratories. A third reason for implementing SPE was the need for the determination of polar degradation products which are fairly soluble in water and therefore less amenable to solvent extraction [2,14].

Trace-enrichment techniques are commonly used off-line. Pretreatment steps are therefore clearly separated from the chromatographic separation. Solid-phase extraction can be also coupled on-line to the chromatographic separation [15–18]. However, liquid chromatography has gained in popularity these recent years owing to its suitability for the determination of polar or non-polar and/or thermodegradable compounds without any derivatisation step, and also owing to its automation potential. Many multiresidue analysis of pesticides and other pollutants have been reported in the literature [19–21].

Automatic devices coupling on-line the sample pretreatment by solid-phase extraction

and the liquid chromatographic separation have been introduced now by some companies. It is certainly a fast modern and reliable approach for monitoring traces of organic in water since it is a completely automated method and there is no sample manipulation between the sample percolation and the analysis.

This chapter is focused on sample handling techniques based on liquid–liquid or liquid–solid extraction procedures with emphasis on the reduction of consumption of organic solvents, and on the on-line coupling of solid-phase extraction with liquid chromatography.

1.2 LIQUID–LIQUID EXTRACTION PROCEDURES

1.2.1 Basic parameters

Liquid–liquid extraction is based on the partition of organic compounds between the aqueous sample and an immiscible organic solvent. The efficiency of an extracting solvent depends on the affinity of the compound for this solvent as measured by the partition coefficient, on the ratio of volumes of each phase and on the number of extraction steps. Solvent selection for the extraction of environmental samples is related to analyte nature [22–25]. Non-polar or slightly polar solvents are generally chosen. Hexane and cyclohexane are typical solvents for extracting aliphatic hydrocarbons and other non-polar contaminants such as organichlorinated or organophosphorus pesticides [26]. Dichloromethane and chloroform are certainly the most common solvents for extracting non-polar to medium polarity organic contaminants. The large selection of available pure solvents, providing a wide range of solubility and selectivity properties, is often claimed as an inherent advantage of LLE techniques. In fact, each solvent is seldom totally specific toward a class of compounds and LLE is mainly used for the wide spectrum of compounds extracted. The so-called lipidic fraction is obtained by extraction with chloroform and contains many organic compounds such as alkanes, aliphatic and aromatic hydrocarbons, alcohols, fatty acids, sterols. From 1 litre of water sample and three extractions with a total volume of 200 ml of dichloromethane, average extraction recoveries obtained for about 30 commonly applied medium-polarity pesticides are between 60% and 90% [27]. The extraction recoveries depend on the spiking level and are higher when samples are spiked with 200 ng/l instead of 50 ng/l. The recoveries may also be different when measured in spiked pure water samples or in real samples.

LLE can be performed simply, and batchwise, using separated funnels. The partition coefficient should be therefore large because there is a practical limit to the phase volume ratio and the number of extractions. When the partition coefficient is small and the sample very diluted, a large volume must be handled and continuous liquid–liquid extractors should be used. Extractions take therefore several hours. Such extractors have been described in the literature [23,28–30].

The partition coefficient may be increased by adjusting the pH to prevent ionisation of acids or bases or by forming ion pairs or hydrophobic complexes (with metal ions for instance). The solubility of analytes in the aqueous phase can be reduced by adding salts. Fractionation of sample into acidic, basic and neutral fractions can be obtained with subsequent extractions at different pH [31]. A typical scheme is represented in Fig. 1.1. This type of fractionation was applied for the determination of pentachlorophenol in sewage sludge and contaminated waters. No further clean-up of the acidic fraction was
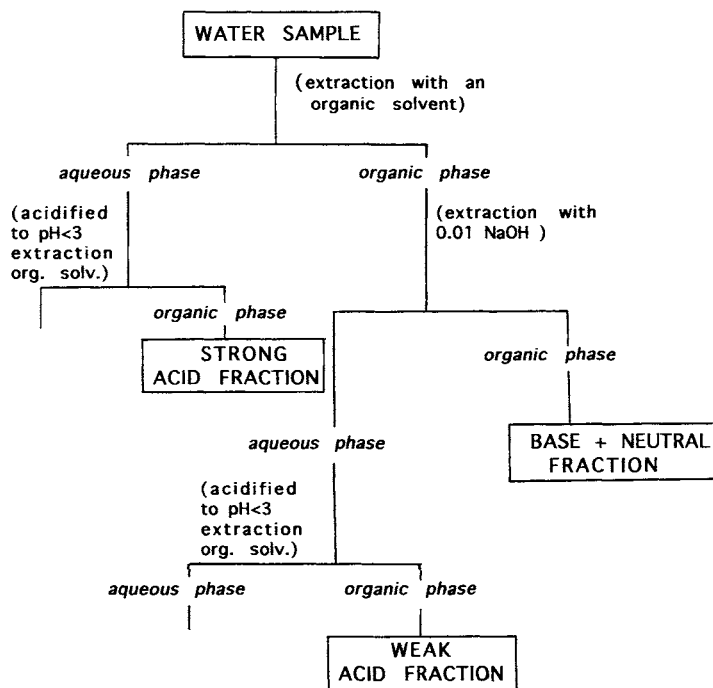
Fig. 1.1. Typical scheme for fractionating water samples into acidic, basic and neutral extracts.

needed and pentachlorophenol was determined by GC using electron-capture detection after simple methylation [32].

It is difficult to compare recoveries obtained by different laboratories because extraction conditions (pH, phase ratio, number and time-length of extractions, salinity, etc.) are generally different. Sample volumes can be very high up to 200 l and more [33]. Sample volumes of 50 l of surface water [34] or 20 l of sea water [35] allow the determination of 5 ng/l of alkanes. When using a specific detection, the sample volume can be lower: 2 ng/l of polyaromatics were determined from 1 litre of river water using liquid chromatography and fluorescence detection [29]. Chlorophenols were determined from 100 ml of sea-water below the 10 ng/l level with electron capture detection coupled to gas chromatography [36].

The LLE of relatively polar and water-soluble organic compounds is in general difficult. The recovery obtained from 1 litre of water with dichloromethane is 90% for atrazine but lower for its more polar degradation extracted products, deisopropyl- (16%) deethyl- (46%) and hydroxy-atrazine (46%) [37]. Some transformation products are more polar than the parent molecules (aldicarb sulfone and sufoxide for instance) and were extracted with recoveries lower than 50% from 1 l of water and using dichloromethane [14].

1.2.2 Trends for reducing solvent consumption: micro-liquid–liquid extractions

In the EPA methods for the determination of pesticides in water based on LLE, the

typical sample volume is 1 litre (methods 507, 508 and 515.1), except for methods 504 and 505 which involve a microextraction [38–40]. It is important to note the trends shown by the EPA for reducing the consumption of organic solvents by carrying out micro-extractions. Only 2 ml of dichloromethane is required in methods 504 and 505. Such micro-LLE can allow quantification at the 0.1 μg/l level for some specific compounds, as shown in method 504. Atrazine and six acetanilide herbicides and metabolites were rapidly determined in ground water in the 0.1–2.5 μg/l range using a one-step extraction of water samples (60 ml) with 1 ml of hexane, followed by direct analysis of extracts using capillary GC with nitrogen-phosphorus detection or combined GC–MS [41]. A rapid micro-LLE was also described for trace analysis of organic contaminants in ground- and drinking water [42]. Another example is the determination of organochlorine and pyrethroid insecticides extracted by 10 ml of hexane for 15 ml of water samples and further concentrated to 1 ml. After an automated clean-up, such a micro-extraction allows one to analyse a group of eighteen organochlorine pesticides and the main pyrethroid insecticides in surface waters at the ng/l level [43].

1.2.3 Concentration procedures

In general, LLE results in the extraction of the sample in a relative large volume of solvent which can be concentrated using a rotary evaporator or a Kuderna–Danish evaporative concentrator or some other automated evaporative concentrator down to a few millilitres. Further concentration down to a few hundreds of microlitres can be obtained by passing a gentle stream of pure gas over the surface of the extract contained in a small conical-type vessel. The solvent-evaporation method is slow and has a risk of contamination. Micro-extractors have been described and have the advantage of avoiding the further concentration of organic solvents [22,44].

1.2.4 Advantages and drawbacks

The main advantages of LLE are its simplicity and its requirement for simple and non-expensive equipment. However, it is not free from practical problems such as the formation of emulsions which are sometimes difficult to be broken up. The evaporation of large solvent volumes and the disposal of toxic and often inflammable solvents, are inherent to the method. The LLE requires several sample-handling steps and contamination and loss have to be avoided at every step. There is a risk of exposure of the chemists to toxic solvents or vapours. The glassware equipment must be carefully washed up and stored under rigorous conditions. The organic solvents must be very pure and expensive pesticide-grade solvents should always be used when determining traces of pollutants in water,.

Carrying out LLE in the field is not easy and large water samples are usually transported and stored in laboratories. Automation of the whole procedure of extraction and concentration requires the use of expensive robots, so it is typically an off-line procedure. Loss during transfer and evaporation steps always occurs, although to a small extent. Standards are therefore often added before LLE and then the recoveries calculated from standard peaks by supposing that losses are similar for solutes and standards. Solubilisation of the standards in the samples should be assessed carefully. Losses due to adsorption on vessels are frequently encountered, especially for apolar solutes.

All these arguments explain why liquid–liquid extractions are often described as tedious, time-consuming and costly.

1.3 SUPPORTED LIQUID MEMBRANE EXTRACTION PROCEDURES

1.3.1 Description and basic parameters

Sample preparation by means of liquid membrane extraction combines the selectivity and enrichment possible with LLE with the capability of efficient removal of disturbing matrix constituents, and requires only a few millilitres of organic solvents [45,46]. The SLM techniques involve the use of a porous PTFE membrane separating two aqueous solutions. The membrane is impregnated with an organic solvent and mounted between two flat blocks in which grooves are machined, forming a flow channel on each side of the membrane. Other configurations are also possible, e.g. utilising a hollow fibre impregnated with an organic solvent [47–49]. The device is connected to a flow system, permitting aqueous solutions to be independently pumped through each of the channels. By proper selection of these solutions, compounds can be selectively extracted from one solution (the donor) into the organic membrane liquid and subsequently extracted into the other solution (the acceptor). The compounds of interest, usually present in ionic form in the donor, together with a suitable reagent, form a non-ionic species which can be extracted into the organic membrane phase. The non-ionic species are then transported through the membrane by diffusing into the aqueous acceptor phase. There, the chemical conditions should be such that the analyte will be converted into a non-extractable form, preventing their re-extraction into the organic phase again. In a typical arrangement the acceptor phase is stagnant and can trap a considerable fraction of the analyte of interest which was originally present in the large volume of sample solution pumped through the donor channel.

As an example, the acidic compounds in water are extracted in protonated form from the acidified water sample which is pumped through the donor channel. After passage through the membrane the acids are trapped in a sufficiently alkaline stagnant acceptor phase. In this way, an enrichment factor of several hundreds can easily be attained, with efficient separation from humic substances and other disturbing species in the water samples. Basic compounds can be extracted from basic donor solutions and trapped in more acidic acceptor solutions. Various charged species can be extracted as uncharged complexes or ion-pairs and trapped on the acceptor side by breaking these complexes in suitable ways.

The selectivity of the extraction process depends primarily on the possibility of transferring the analytes of interest between active and inactive forms in the required sequence, without making the same transfers for interfering compounds. The chemistry of the process is important, and compounds which can be handled by the SLM techniques are mainly ionisable analytes in the pH range 1–14 and compounds which can form complexes.

1.3.2 Environmental applications

Environmental applications include the extraction of organic acids in manure and soil [50,51], aliphatic amines in ambient air and rainwater [52], chlorinated phenols in water

[53], chloroaniline in surface and waste water [54], the determination of various acidic herbicides such as chlorophenoxy-acid, sulfonylurea and chlorotriazine herbicides in water [55–60], cationic tensides [61] and the trace enrichment of metals [62]. The SLM technique has been used both off-line and on-line with direct connection to liquid chromatography, using a flow system in which the extracted sample is pumped into the injection loop of the liquid chromatograph [55,58]. A field sampling technique for acidic herbicides has been described where an integrated and specific sampling during 24 h is performed automatically [56,57].

1.3.3 Advantages and drawbacks

The SLM extraction can be used to selectively extract certain classes of compounds while other classes are not extracted. Environmental samples containing high concentration of matrix constituents such as humic substances and colloidal particles can be processed over long periods. The enrichment is made early in the analytical procedure, which facilitates further operations. The process is performed in a closed flow system, which also minimises the risk of contamination and can facilitate the handling of dangerous samples. The use of organic solvent is minimal, just a few millilitres to impregnate the membrane. Extraction recoveries can be close to 100% and large enrichment factors can be obtained. In field sampling, several litres can be processed and enriched into a small volume, e.g. 1 ml, of acceptor solution, leading to enrichment factors up to more than 1000. The flow system allows the technique to be easily automated and directly coupled with a subsequent clean-up treatment, if necessary, and with the final analytical chromatographic step. Then, the technique can be integrated with the sampling.

The main drawback is the time of sampling, since percolation of the sample cannot be performed with a high flow rate, and the limitation of applications to analytes having ionisation or complexation properties.

1.4 SOLID-PHASE EXTRACTION

Solid-phase extraction is today the method of choice for carrying out simultaneously the extraction and concentration of many organic compounds in aqueous samples. Although SPE was introduced twenty years ago, its acceptation in environmental analysis is rather recent and occurred these last 6 or 7 years. The availability of cleaner and more reproducible sorbents than in the past has certainly helped in its acceptance by regulatory agencies. Other reasons are the large choice of sorbents, packed in cartridges or enmeshed in filtration disks, some of them now having the capacity of trapping polar analytes and the development of automatic devices. SPE is included in official methods established by the U.S. Environmental Protection Agency (USEPA) for the determination of various organic compounds in drinking water (phthalates, tetrachlorodibenzo-*p*-dioxins, chlorinated acids, polycyclic aromatic hydrocarbons, benzidines, nitrogen-containing pesticides, organochlorinated pesticides, haloacetic acids, carbonyl compounds, diquat and paraquat) and in waste water (phenoxy-acid herbicides, organohalide pesticides, organophosphorus pesticides, organochlorine pesticides, polychlorinated biphenyls, benzidines and nitrogen containing pesticides).

However, this technique appears less straightforward than LLE, because there is a large

choice of sorbents and because recoveries depend on the sample volume. In fact, SPE is simple when one considers it is based on the well established separation principles of liquid chromatography.

1.4.1 Description

SPE can be used off-line, the sample preparation being completely separated from the subsequent chromatographic analysis, or on-line, by direct connection to the chromatographic system.

1.4.1.1 Off-line methods

In off-line methodologies, the samples are percolated through a sorbent, packed in disposable columns or cartridges, or enmeshed in an inert matrix of a membrane-based extraction disk. The syringe-barrel and/or cartridge types are still the most popular format and are available by most of manufacturers under various trade names such as Sulpelclean, Quick-Sep, Bond-Elut, Baker-Bond, Sep-Pack, Extra-Sep, Hyper-Sep, Extra-Clean, Isolute, etc. The sorbent bed varies from 100 to 2000 mg and is retained between two porous frits. The design may vary in order to be robot-compatible. Reservoirs have been adapted in order to increase the sample volume. As a general rule, in addition to the use of cleaner phases, the manufacturers have made efforts to provide high-purity devices with low extractable contents using medical-grade polypropylene and polyethylene for the cartridge body and frits. Limitations of packed SPE conventional cartridges include restricted flow rates and plugging of the top frit when handling water containing suspended solids such as surface water or wastewater. Therefore, the percolation of samples can take a long time for a typical volume of 500 ml if the sample has not been carefully filtered before. In order to avoid previous filtration and clogging, various approaches have been investigated to overcome the flow limitation. Depth filters containing diatomaceous earth have been available as accessories by some companies. The trends are now to integrate filters in the SPE cartridges.

Single samples can be processed by attaching a syringe to the SPE columns or reservoir for application and elution. Sample can be also aspirated through the column by vacuum. The granulometry of the bed-packing is between 30 and 75 μm so that high flow rate can be applied. Another method of application is to use centrifugation by inserting SPE cartridges into an appropriately centrifugation tube. Various vacuum manifolds allow batches of up to 24 samples to be prepared simultaneously. The application of samples and solvents in a SPE process can be thus performed semi-automatically, with no risk of sample contamination. Some reservoirs are compatible with the Zymark laboratory robot and the sequence can be totally automated.

A typical SPE sequence involves four steps. First, the SPE columns is prepared to receive a sample, by activation or wetting with a suitable solvent, and by conditioning with water. Then, the aqueous sample is applied, and, often, analytes of interest are trapped together with other components (interferences) of sample matrix. Then, some of these interferences can be removed by application of a washing solvent in the so-called clean-up step which will be examined more in details later. In the last step, elution of the concen-

trated analytes is performed by application of a small column of organic solvent, which can be further gently evaporated to increase the enrichment factor.

The SPE disks have been introduced in the early 1990s and their use is particularly easy [63]. The first disks contained the sorbent enmeshed in a Teflon matrix. Recently, new disks have become available with the sorbent in a glass fibre matrix. They are thicker and more rigid and provide faster flow rates than Teflon disks and may require no supporting device [64]. SPE disks have been tested for various groups of compounds including pesticides, organotins, and phthalates [65–77]. The USEPA has approved various methods based on the use of SPE disks containing either C_{18} silica or a styrene divinylbenzene (SDB) sorbent. The disks are available with diameter and size similar to liquid chromatographic solvent filters (47 and 90 mm). The membrane is placed in a filtration apparatus attached to a water-aspirator vacuum source, the disk is conditioned with 10 ml of methanol and 10 ml of organic-free water, and the water sample is filtered through it. Then the extraction funnel and frit assembly is transferred to a second vacuum filtration flask containing a test-tube. A 5 ml aliquot of the eluting organic solvent is then drawn through the membrane, with the vacuum being interrupted at this point to allow it to soak the disk for several minutes. This is generally repeated with another 5 ml aliquot. Apparatus have been developed which gives better performance over the whole procedure (Separex from J.T. Baker, for example).

The main advantage of using SPE membrane disks rather than SPE cartridges is the increased productivity permitted by the relatively high flow-rates. In general, the time required for the isolation of the various pollutants using disks is half of that using cartridges (30 vs. 60 min for 1 l of water). When determining surface- or sea water samples, one is recommended to prefilter the samples through 0.45 μm PTFE filters. As the prefiltration can be connected on-line with an Empore disk, the time required for handling water containing suspended matters is much shorter. An Empore aid filter is available which can placed on top of extraction disks to a depth of about one cm. It is made of glass beads with a typical diameter of 40 μm and is non-porous, inert and inhibits the migration of suspended matter to the surface of the disk. This method also has the advantage of being well adapted to analysing the partitioning between the dissolved and the suspended phase by analysing the content of the disk and the glass or PTFE filter, respectively. J.T. Baker has introduced new laminar disks known as Speedisks which consist in a thin bed of microparticles supported in a laminar structure in a preassembled disk. The percolation of 1 l of surface water without any previous filtration takes less than 5 min [21].

Very recently, disks have been introduced in rigid SPE cartridges, known as SPEC microcolumns (Solid-Phase Extraction Concentrator). Their main advantage is the unique rigid disk structure which avoids the creation of voids and channels which can occur in the packed beds of the conventional SPE cartridges. Since there are no frits, the void volume is very small, so the washing and desorption steps may be accomplished very efficiently with small quantities of reagent. However, the amount of sorbent in the available SPEC is 5–56 mg, depending on the diameter and thickness of the disk, which can limit the sample volume and therefore not allow trace-analysis at low levels.

Compared with LLE-based sample preparation, the off-line SPE offers reduced processing times and substantial solvent savings. Percolation of samples can be performed in the field and good storage of adsorbed analytes is generally observed [71,73–77]. The problem

of transport and storage of voluminous samples is avoided, which is especially interesting when samples have to be taken at remote sites. Automation is possible using robotics or special sample preparation units that sequentially extracts samples and clean them up for automatic injections. The possibility exists for some of these devices for automatic injection of an aliquot of the final extract into the chromatographic system. Examples are the ASPEC from Gilson, Microlab from Hamilton, AutoTrace and RapidTrace from Zymark. Nevertheless, a certain amount of tedious labour remains and off-line procedures have the inherent disadvantages of loss in sensitivity owing to the injection of an aliquot, losses in the evaporation step, and some risks of contamination, so that internal standards are required.

1.4.1.2 On-line methods

On-line coupling of SPE sample preparation to GC or LC separation avoids many of the problems mentioned above. On-line approaches coupling SPE to LC are particularly easy to perform in any laboratory and are known as column switching or precolumn technology, or on-line multidimensional chromatography. They have been extensively developed by Frei and co-workers more than 10 years ago [78,79]. A typical scheme [80] for an on-line procedure coupled to liquid chromatography is show in Fig. 1.2. The extraction precolumn is placed in the sample-loop position of a six-port liquid switching valve. After sample conditioning, application, and eventual cleaning via a low-cost pump, the precolumn is coupled to an analytical column by switching the valve to the inject position. The absorbed compounds are then eluted directly from the precolumn to the analytical column by a suitable mobile phase which also enables the chromatographic separation of trapped compounds. One can expect more accurate quantitative results as there is no sample manipulation between preconcentration an analysis. Automation is easy and several devices are now commercialised (OSP-2 from Merck, Prospekt form Spark Holland, Aspec XL from Gilson). This apparatus improved productivity since the next sample is automatically prepared while the previous sample is being analysed.

In contrast with off-line SPE, the entire sample is transferred and analysed, allowing the handling of smaller sample volumes. A more detailed description of on-line technique is given in the last part of this chapter.

1.4.2 Basic principles

The chemistry and principles are essentially identical for both off-line and on-line SPE. To a first approximation, SPE can be considered as a simple chromatographic process, the sorbent being the stationary phase. The mobile phase is the water of the aqueous sample during the extraction step or the organic solvent during the desorption step. Retention of organic compounds occurs to the extent that they are not eluted by water during the extraction step. Reversed-phase materials are widely used because, in reversed phase chromatography, water is the less eluting mobile phase for neutral organic compounds. The main sorbents that can be used for retaining organic compounds in aqueous media are reported in Table 1.1, with the corresponding separation mechanisms involved, the nature of the elution solvent, the characteristics of analyte concentrated and some applications. The highest enrichment factors are obtained when there is a high retention of analyte by
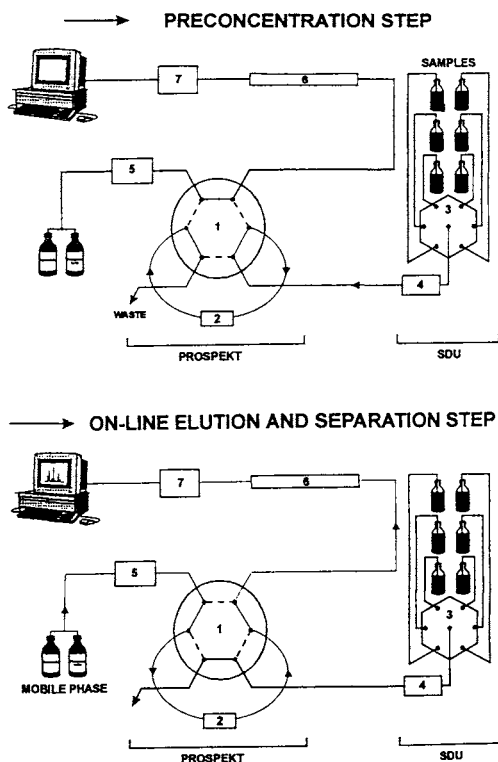
Fig. 1.2. On-line set-up. 1, LC switching valve; 2, precolumn; 3, switching valve of the solvent delivery unit; 4, preconcentration pump; 5, LC pump; 6, analytical column; 7, detector. From [16].

water and a low retention by the desorbing organic solvent. With pure organic solvents, desorption occurs for volume close to the void volume of the column. From a practical point of view, to obtain high enrichment factors one should select the sorbent that gives the highest retention of analytes in water. Breakthrough of solutes occurs when they are no longer retained by the sorbent. Overloading of the capacity of the sorbent can also be responsible for breakthrough of analytes [78]. In practical environmental analyses of organic pollutants, where concentrations are typically of the μg/l order, it is rather unlikely that breakthrough will occur by overloading of the sorbent capacity.

1.4.2.1 Breakthrough volume

Fig. 1.3 represents a breakthrough curve obtained by monitoring the UV signal of the effluent from an extraction column. A solution of water spiked with an organic compound at trace level and having a UV absorbance A_0 is percolated through a SPE column. Whilst the compound is retained by the sorbent, it is absent from the effluent which will have a UV absorbance of zero. For a volume V_b, usually defined as 1% of the initial absorbance A_0 [80], a frontal or breakthrough curve is recorded, and after a volume V_m, usually defined as 99% of the initial absorbance, the eluate has the same composition as that of the spiked

TABLE 1.1

DIFFERENT SORBENTS USED FOR SOLID-PHASE EXTRACTION AND ALLOWING PRECONCENTRATION OF ANALYTE FROM A SUFFICIENT WATER SAMPLE VOLUME FOR TRACE LEVEL DETERMINATION; INVOLVED CHROMATOGRAPHIC SEPARATION MECHANISM, CHARACTERISTICS OF ANALYTES AND SOME ENVIRONMENTAL APPLICATIONS

Sorbent	Separation mechanism	Elution solvent	Nature of analyte	Environmental applications
Octadecyl-/octyl-bonded silicas	Reversed-phase	Organic solvent	Non-polar and weakly polar	AHs, PAHs, PNAs, PCBs, organophosphorus and organochlorine pesticides, alkylbenzenes, polychlorophenols, phthalates, esters, polychloroanilines, apolar herbicides, fatty acids, aminoazobenzene, aminoanthraquinone, etc.
Porous styrene-divinylbenzene copolymers	Reversed-phase	Organic solvent	Non-polar to polar aromatic	Phenol, chlorophenol, aniline, chloroaniline, polar herbicides (phenoxyacids, triazines, phenylureas), etc.
Graphitised carbon	Reversed-phase	Organic solvent	Non-polar to very polar aromatics	Alcohols, nitrophenols, aminophenols, polar herbicides and metabolites, polar aromatic derivatives
Silica- and polymer-based ion-exchangers	Ion- exchange	Water (pH adjusted)	Cationic and anionic organics	Phenol, nitrilotriacetic acid, phenoxyacids, phenylenediamines, aniline and polar derivatives, sulfonic acids, phthalic acids, aminophenol, etc.
Metal-loaded	Ligand-exchange sorbents	Complexing aqueous solution	Metal-complexation property	Aniline derivatives, amino acids, 2-mercaptobenzimidazole, carboxylic acids, buturon, etc.
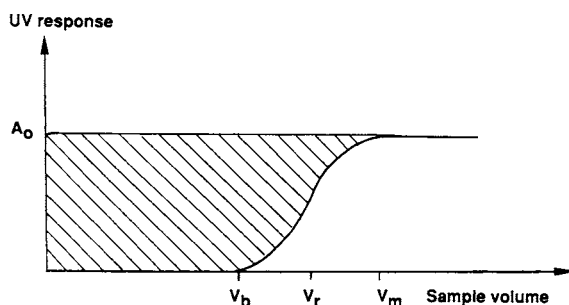
Fig. 1.3. Breakthrough curve obtained after percolation of a spiked water sample with a UV absorbance A_o through a SPE precolumn. From [80].

water solution. Under ideal conditions, the curve has a bi-logarithmic shape and the inflection point is the retention volume of the solute eluted by pure water, V_r, if the column is not overloaded. The quantity V_b corresponds to the sample volume that can be percolated with no breakthrough of analyte.

In trace analysis, the amount of extracted analyte available for detection has to be maximised: it is obtained for a sample volume of V_m (hatched area in Fig. 1.3). Percolation of a higher volume than V_m does not increase the amount extracted. The breakthrough volume, which can be estimated in a first approximation from retention volume in water [78,80–84], is the most critical parameter for preconcentration. Knowing the concentration limit required (0.05 µg/l for instance) and the absolute detection limit of the chromatographic detection (25 ng injected for instance), one can easily therefore calculate the minimum sample volume necessary (500 ml in the cited example), and obtained therefore a magnitude order of the minimum retention volume, V_r, required.

1.4.2.2 Recoveries

Recovery is defined as the ratio between the amount extracted and the amount percolated. As can be seen in Fig. 1.3, a theoretical 100% recovery can be obtained only for a sample volume equal or lower to V_b. The maximal amount does not correspond to a 100% recovery and is reached for a sample volume equal to V_m. Therefore, the recovery in SPE depends both on the sample volume percolated and on the V_b value which is related to the chromatographic retention volume in water, V_r, and then to the nature and the amount of sorbent. This explains why recovery values can be compared only if sample volumes and amounts of sorbent are known. In SPE, it is always possible to show examples with recoveries of 100% by decreasing the sample volume below the corresponding V_b. A simple calculation indicates if the handling of this volume would allow the required detection or not. Many intercomparisons between LLE and SPE have been made without taking this parameter into account.

If recoveries are too low for detection, the only remedy is to increase V_b (or V_r), which can be obtained by increasing the amount of sorbent or choosing another sorbent giving a higher retention in water for analytes of interest.

UV response
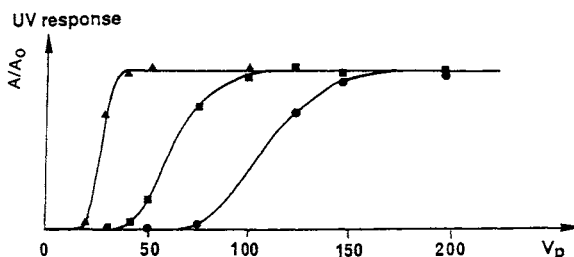
Fig. 1.4. Experimental breakthrough curves recorded with a 1 × 0.21 cm i.d; precolumn packed with RP-18 silica. Samples: solution spiked with 100 μg/l of (▲) simazine, (■) atrazine and (●) linuron. From [80].

1.4.2.3 Experimental determination of breakthrough volumes and recoveries

Recording breakthrough curves is time consuming and reading V_b at the 1% level is neither easy nor always accurate [2,6,78,80–85]. The sample should be spiked at a trace level in order not to overload the sorbent capacity, and the UV signal should be monitored at very low absorbencies, which may lead to problems with baseline stability or noise. The Fig. 1.4 shows experimental breakthrough curves obtained for three herbicides with a 10 × 2.1 mm i.d. precolumn packed with C_{18} silica. The breakthrough curves are different, and the more retained the compound is, the larger volume the curve is spread over, because of the low plate number of the precolumn. The front corresponding to linuron spreads over nearly 100 ml from a V_b value of 70 ml to a V_m value of 165 ml. First, the determination of V_b at 1% of the initial absorbance on the front curves cannot be accurate when the front is not sharp. The second point is that if no breakthrough is wanted for a 100% recovery, the percolated volume has to be lower than 70 ml. Nevertheless, raising the percolated volume to 165 ml considerably increases the amount preconcentrated by nearly to 50%. The corresponding recovery is then below 100%, but overcoming the breakthrough volume may sometimes be interesting when traces of organic compounds have to be determined in water samples having relatively low organic contamination. Of course, the same situation occurs for some of the analytes when many solutes of different polarity are to be determined together.

A faster method for estimating breakthrough volumes and recoveries has been developed [80,83]. It is easily performed with the on-line apparatus, but can also be carried out using off-line preconcentration [8,86,87]. It consists of preconcentrating water samples of increasing volumes, each containing the same amount of analytes, and then measuring the peak-areas or heights eluted on-line from a precolumn, or off-line from a cartridge or disk. As the sample volume increases, the analyte concentration decreases, provided breakthrough does not occur: the amount preconcentrated remains constant and the peak areas in the on-line chromatograms following desorption are constant. When breakthrough occurs, the amount extracted is reduced, and the desorption peak-area or height decreases. The corresponding recoveries can be calculated by dividing the peak areas obtained after breakthrough by those obtained before. This is shown in Fig. 1.5. An advantage of this method is that the V_b values of several compounds can be estimated simultaneously by
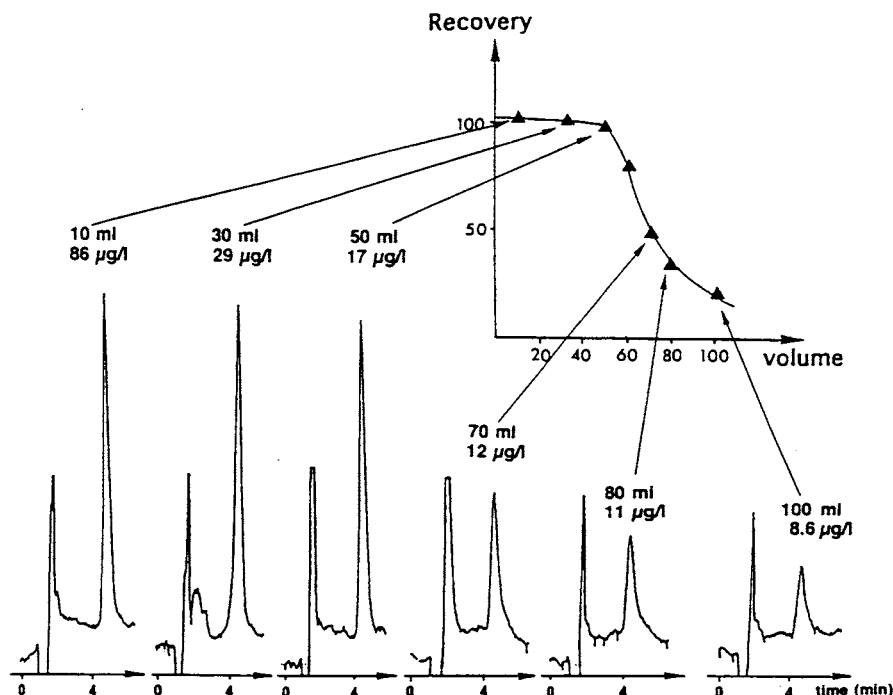
Fig. 1.5. Experimental determination of the breakthrough volume and corresponding recovery. (From [8] with permission). Different sample volumes, containing the same amount of cyanuric acid (0.86 μg), are percolated through a 1×0.46 cm i.d. precolumn packed with porous graphitic carbon, PGC (10 μm). The chromatograms correspond to the on-line elution of each sample using a 10×0.46 cm i.d. analytical column prepacked with PGC (Hypercarb) using a mobile phase containing 30% methanol and 70% 0.05 M sodium phosphate at pH 7; the flow rate is 1 ml/min; UV detection at 220 nm. Recoveries are calculated from the ratio of peak areas. The sample volume and the corresponding concentration are indicated on each chromatogram.

preconcentration and analysis under the real experimental conditions of unknown samples, via the whole off-line or on-line procedure.

1.4.2.4 Prediction of breakthrough volumes and recoveries from LC data

The breakthrough volume can be estimated using V_r, which is related to chromatographic data and cartridge or precolumn characteristics by the relation

$$V_r = V_0(1 + K_w) \tag{1}$$

where V_0 is the void volume of the precolumn or the cartridge and k_w is the retention factor of the solute eluted by water. V_0 can be calculated from the porosity of the sorbent (ε) and the geometric volume (V_c) of the precolumn or sorbent bed in the cartridge or disk ($V_0 = (\varepsilon V_c)$. Most of the reversed-phase sorbents used in cartridges have an average porosity between 0.65 and 0.70. With an average density of 0.6 g/ml for the C_{18} silica used in cartridges, V_0 is estimated as 0.12 ml per 100 mg of sorbent.

The V_b values read at 1% of the initial absorbance can be also calculated from the k_w values as developed below, because V_r is linked to V_b by the relation

$$V_b = V_r - 2.3\sigma_v \tag{2}$$

where σ_v is the standard deviation depending on the axial dispersion along the bed of particles in the precolumn or cartridge. V_b is therefore controlled by retention and kinetic parameters [2,78,85,88–92]. The σ_v term can be calculated if the number of theoretical plates, N, of the precolumn or cartridge is known by the relation

$$\sigma_v = \left(V_0/\sqrt{N}\right)\left(1 + K_w\right) \tag{3}$$

N can be directly measured with precolumns because the on-line set-up can allow the recording of breakthrough curve or of the elution peaks by direct injection onto a precolumn [80]. It is much more difficult to measure the efficiency of a SPE cartridge or that of an extraction disk, so that N has to be estimated. Miller and Poole [88] have studied the kinetic and retention properties of an SPE cartridge packed with 500 mg of C_{18} silica and they measured an average of 20 theoretical plates for a flow rate of 5 ml/min.

The breakthrough curves have been modelled according to the relations described above and the mathematical representation of the breakthrough curves as function of the percolated volume. In order to compare with experimental curves, the effect of log k_w on the shape of the curves has been modelled for a sorbent having a void volume of 0.54 ml (which corresponds to an extraction disk containing 450 mg of sorbent) and with 20 plates [6]. The corresponding theoretical recovery curves are represented in Fig. 1.6. First, the more polar the analytes are, the sharper the fronts are. These curves are much more relevant from a practical point of view than breakthrough curves. On one hand, they show that breakthrough can be overloaded to a great extent, with small losses in recoveries, for compounds with high log k_w values. For example, a compound characterized by a
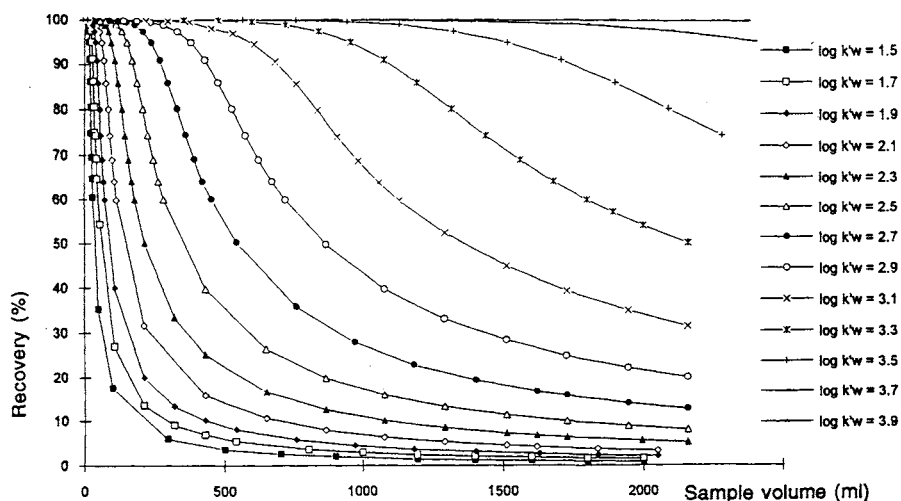
Fig. 1.6. Effect of the log k_w values of the analyte on theoretical recovery curves assuming 20 plates in the cartridge or disk. From [6].

log k_w of 2.9 (calculated V_r value of 430 ml), has a breakthrough volume of 210 ml, but, the theoretical recovery value obtained with a sample volume of 500 ml is still around 85%. Only compounds with log k_w lower than 2.5 will be extracted with recoveries lower than 50% with a 500-ml sample volume. On another hand, when log k_w is lower than 2, the recovery decreases rapidly as soon as overloading of V_b occurs.

Since it is difficult to estimate N especially in cartridges, it appeared necessary to evaluate the effect of this parameter on recovery curves. The predicted recovery curves were very similar for a compound characterised by a log k_w value of 2.7 using a sorbent containing 10 and 20 plates and having a V_0 value of 0.54 ml. With 10 plates the V_b value is 100 ml. If N is underestimated and equal to 20 then V_b is 150 ml. However, with a sample volume of 150 ml and 10 plates, the recovery is still 98%. Therefore, the error is of the order of the experimental ones.

Depending on the sample volume required, these theoretical curves indicate the necessary k_w for obtaining a recovery in the range 90-100%, so that k_w is the most relevant parameter to be known for prediction. The practical problem is then to select a sorbent able to provide the required k_w value. This also explains why comparison of the sorbents have been made using k_w values from LC data [93–99]. Several methods exists for their extrapolation or prediction, depending on the retention mechanism between analytes and sorbents.

1.4.2.5 Agreement between predicted and experimental curves

The agreement between experimental and theoretical recoveries curve has been obtained for a set of polar pesticides, using two types of extraction disks containing 450 mg of C_{18} silica and 450 mg of styrene divinylbenzene polymer, respectively. The log k_w were extrapolated from k values measured in methanol-water mixture using short column in order to have experimental values with water rich mobile phases. Fig. 1.7 reports the experimental variations of the recoveries with the sample volume with the calculated curves for oxamyl using a C_{18} and a SDB disk, respectively. Taking account of the fact that recoveries are obtained with average standard deviations of 10% due to the different steps of the SPE sequence, the agreement between calculated and experimental curves is very good.

One should also mention the great difference in V_b values obtained using a C_{18} disk and a SDB disk. The advantage of using a sorbent providing a larger retention factor in water is shown in Fig. 1.7 since for oxamyl, a recovery of 25% is observed for a 100-ml sample with a C_{18} disk and for a 1000-ml sample with a SDB disk.

1.4.3 Sorbent selection

1.4.3.1 n-Alkylsilicas

For many years most of the off-line SPE procedures for the handling of environmental samples have been achieved using C_{18} silicas and to a less extent C_8 silicas. They are very pressure resistant and are available in various granulometry, typically from 3 μm to 200 μm. Their main drawback is their bad stability in very acidic and basic media, which limits their use in the pH range between 2 and 8. Nevertheless, good reproducibility in retention,
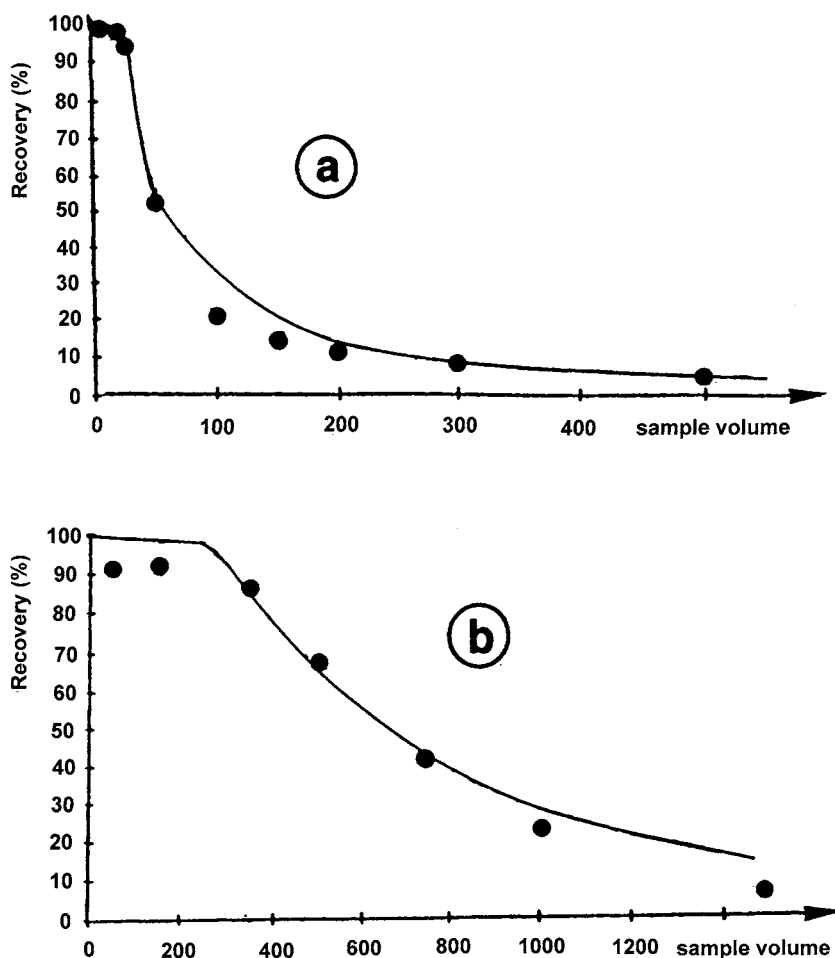
Fig. 1.7. Predicted recovery curves (plain line) obtained for oxamyl with the sample volume and (●) experimental values using (a) a C_{18} Empore disk (J.T. Baker, diameter 47 mm) and (b) a SDB Empore disk (J.T. Baker, diameter 47 mm); LC-grade water sample packed with a constant amount (40 μg) of analyte. From [6].

rapid equilibrium with mobile phases and very few irreversible adsorption of solutes explain their widespread use.

Prediction from measurements of retention factors in water methanol mixtures. In practice, one first needs an approximate value of V_b for selecting a convenient sorbent and the amount of sorbent. Values of k_w are often estimated from chromatographic measurement using C_{18} analytical columns eluted with mobile phase composed of water-methanol mixtures. The advantage of this method is that experimental data are obtained rapidly by measuring the retention factor k of the analyte in methanol-water phases. Over a methanol content in the range 30–90%, the relationship is usually considered as linear. As shown in Fig. 1.8, this has been observed for phenol using a C_{18} silica
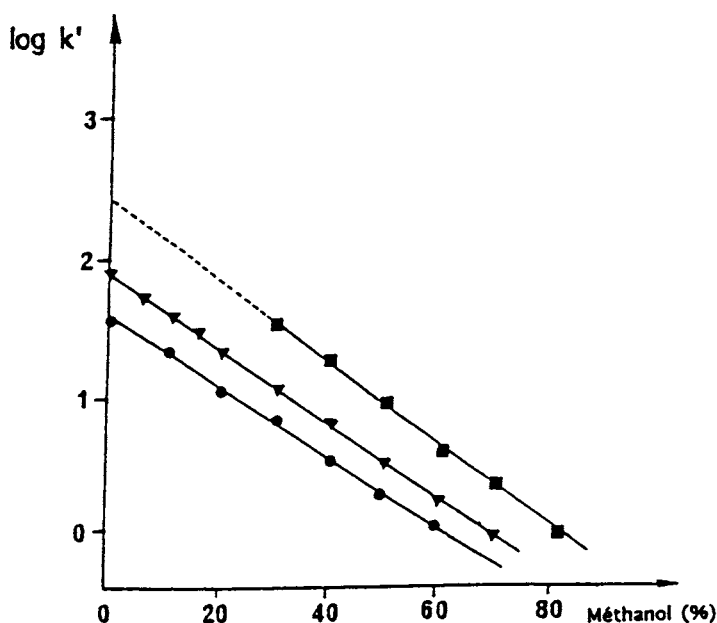
Fig. 1.8. Variation of the retention factor of phenol with the percentage of methanol in the water-methanol mobile phase as measured with (●) C_{18} silica18 silica",4> RP-18 (from Merck), (■) PRP-1 SDB copolymer (from Hamilton) and (▼) Hypercarb PGC (from Shandon). From [6].

and other reversed phase sorbents. Then one can conclude that from rapid measurements with three or four mobile phases containing different methanol concentration, k_w, can be estimated by graphically extrapolating to zero methanol content. However, this relation is known not to be totally linear in water-rich mixture and a better fit has been obtained with a quadratic relationship for some compounds [100]. We have investigated the shape of the curve log k-methanol% for various polar pesticides having different structures and functionalities. Most of the curves have shown that when a wide range of mobile phase is studied polar compounds do not give raise to linear variations, but to quadratic relations [6], so that the value of k_w, extrapolated by the linear relation should be underestimated.

Another important point is that a drastic decrease of breakthrough volume can be observed when percolating water samples containing a small content of methanol or other organic solvent, especially when the relation is a quadratic one. This is a direct consequence of the relationship between log k and the methanol percentage. The addition of 1% by volume of methanol to drinking water samples can produce a 10% decrease in the breakthrough volume. When spiking samples will solutes often dissolved in organic solvent, one has to take care that the final solution should not contain more than 0.1–0.5% of organic solvent.

Relation with the octanol–water partition coefficient. Since the retention mechanism is primarily governed by hydrophobic interactions between the analyte and the carbonaceous moieties of the alkyl chains grafted at the silica surface, a relation has been observed between the retention factors of the analytes and their octanol–water partition coefficient (K_{ow}), which characterises well the hydrophobicity of a compound and plays an important

role in correlating phenomena of physical, chemical, biological and environmental interest [101–103]. Brauman [102] has gathered many log k_w values obtained with different C_{18} silicas using methanol–water as mobile phases and a linear relation was found between the average log k_w values and log K_{ow} for closely related compounds and even for compounds having different polarities and chemical properties. Therefore, k_w values can be approximated without any additional measurements when log K_{ow} values are available.

Table 1.2 reports calculations of retention volumes of apolar to relatively polar organic compounds. The log k_w values have been extrapolated using the relation log k methanol percent from our own results [93] or from values in [102]. The octanol–water partition coefficients have been reported. Calculated V_r volumes have been made for an on-line application using a 1×0.2 cm i.d. precolumn such as those used in automatic devices or a cartridge containing 100 mg of C_{18} sorbent. It can be observed that the V_r volume depends greatly on the hydrophobicity of the solute. For an apolar compounds such as phenanthrene, about 3 litres of sample can be percolated without breakthrough whereas for

TABLE 1.2

OCTANOL–WATER PARTITION COEFFICIENTS (log K_{ow}), log k_w VALUES EXTRAPOLATED FROM THE RELATION log k'-METHANOL PERCENT, AND CALCULATED V_r VOLUME (ml) ON (a) A 1×0.2 cm i.d. ON-LINE PRECOLUMN PACKED WITH C_{18} SILICA OR (b) ON A CARTRIDGE CONTAINING 100 mg OF C_{18} SILICA; SEE TEXT FOR CALCULATION

Compounds	log K_{ow}	log k_w	V_r (a)	V_r (b)
Pyrene	4.88	5	2200	12000
Phenanthrene	4.53	4.42	578	3150
Naphthalene	3.38	3.31	45	245
Ethylbenzene	3.15	3.4	55	300
Toluene	2.76	2.75	12	67
Benzene	2.14	2.2	3.5	20
Fluorobenzene	2.27	2.3	4.5	24
Chlorobenzene	2.84	2.77	13	70
1,2-Dichlorobenzene	3.38	3.39	54	295
Phenol	1.48	1.55	0.8	4.4
2-Chlorophenol	2.16	2.11*	3	15.5
2,6-Dichlorophenol	2.84	2.76*	12	70
3,5-Dichlorophenol	3.56	3.49*	68	370
2,4,5-Trichlorophenol	4.1	3.96*	200	1094
2-Methylphenol	1.93	1.8	1.5	8.5
4-Nitrophenol	1.91	1.84	1.5	8.5
Nitrobenzene	1.84	2.05	2.5	13.5
1,3-Dinitrobenzene	1.49	1.6	0.9	5
Aniline	0.91	1.08	0.3	1.6
4-Nitroaniline	1.39	1.5	0.7	4
4-Chloroaniline	1.83	1.84*	1.5	8.5
Benzylalcohol	1.10	1.40	0.6	3.1
Benzoic acid	1.77	1.90	1.4	10
Benzaldehyde	1.45	1.73*	1.2	6.6
Acetophenone	1.70	1.8	1.4	8

relatively polar compounds such as phenol, aniline, chloroaniline, nitrophenol, breakthrough occurs for less than 10 ml. Disposable cartridges can contain up to 1000 mg so that the calculated volumes can be 10 times higher. Fig. 1.9 shows the relation between extrapolated log k_w from chromatographic measurements and log K_{ow}. One can observe that the relation is good, allowing the determination of log k_w for any compounds if its hydrophobicity constant is known or can be calculated. No measurements is then required.

Few data have been published with regards to polar analytes. Our results indicated a large difference between log K_{ow} and extrapolated log k_w values as close as to real ones [6]. Therefore, log K_{ow} is of limited help for predicting the SPE recoveries, especially for very polar analytes with log K_{ow} below 1.5. It can just serve as a first estimation, knowing that k_w thus predicted can be underestimated by a factor 10–50. For very polar analytes, a more rapid method is certainly to have in the laboratory a 10 or 5 cm-long C_{18} column, and to extrapolate log k_w from k measurement in methanol-water mixture containing as high as possible water content. This is rapid and can be easily performed with autosampler and LC devices.
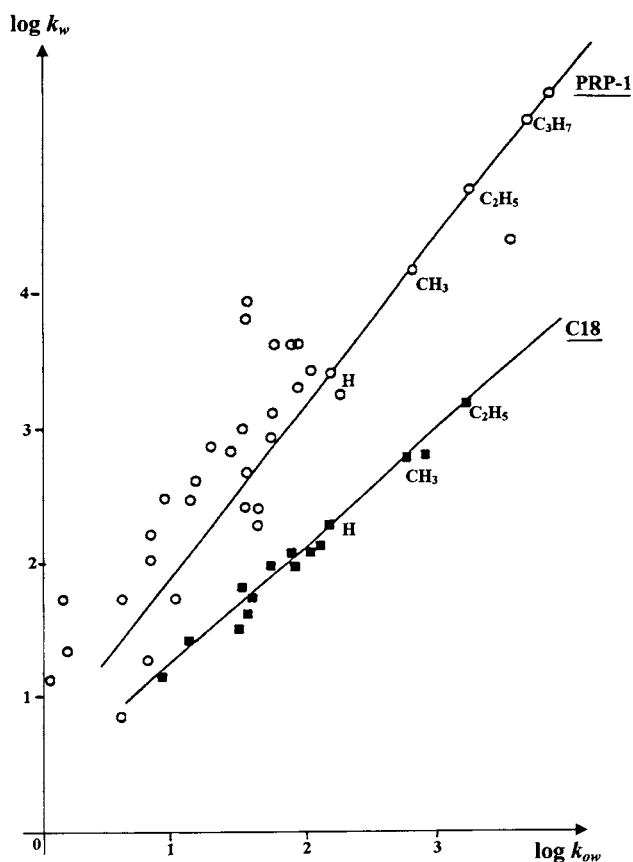
Fig. 1.9. Relationship between the octanol–water partition coefficient (log K_{ow}) and the retention factors (log k_w) estimated or measured in water on C_{18} silicas and PRP-1 SDB sorbent. Adapted from [2].

Differences between C_{18} LC sorbents, standard C_{18} SPE sorbents and C_{18} SPE sorbents designed for polar analytes. Available cartridges are packed with C_{18} silicas having different characteristics and it is well established that in LC, retention differs from one to another C_{18} stationary phase, because retention depends on the number of C_{18} chains bonded at the surface of the silica. Extrapolated log k_w values have been compared using analytical columns prepacked with LiChrosorb RP-18, Bakerbond C_{18} and Sepralyte C_{18}. The standard deviation ranges between 0.05 and 0.12 for a set of analytes with mean values of log k_w between 1.7 and 2.8. These results are in agreement with published works from Braumann et al. [102], when gathering values from different authors and using different C_{18} columns (mainly Nucleosil, Hypersil and LiChrosorb). The values extrapolated have been also compared when using commercial prepacked analytical C_{18} columns and when packing columns with standard sorbents from C_{18} cartridges coming from three manufacturers. Slightly higher k_w values were obtained with sorbents in cartridges and this can be explained by the fact that extraction sorbents has been synthesised from silicas having large specific areas, between 550 and 600 m^2/g. In LC, in order to obtain a better efficiency and a totally apolar material, the trends are to minimise the number of residual silanol groups of the original silica, and for this purpose, a trifunctional silane is used for bonding the n-alkyl chains and an 'end-capping' is carried out with trimethylsilane after bonding [104–109]. Very often, the mobile phase contains an organic solvent which is adsorbed to the stationary phase and ensure a good contact between the solute and the solid. However, the purpose of an extraction is different from LC separations and it was observed that the contact between some polar analytes and a totally hydrophobic C_{18} silica during the SPE process was better when the C_{18} silica was prepared using a monofunctional silane and was not end-capped or contained some polar groups in addition to the alkyl chains. That are the characteristics of various C_{18} SPE cartridges specifically 'designed' for trapping polar analytes (often named C_{18}/OH or polar plus C_{18}). We have compared recoveries obtained for a set of polar carbamates with an on-line system using precolumns of the same size but prepacked with two standard C_{18} sorbents and one C_{18}/OH [6]. Results have shown that recoveries are slightly lower for the C_{18}/OH phase and comparable for the two standard C_{18} sorbents. They were easily explained by the lower carbon content of the C_{18}/OH phase (13.5%) compared with that of the two standard C_{18} sorbents (18%).

Using a monofunctional silane without end-capping provides the highest amount of residual silanol groups [110–113]. A consequence is that secondary interactions such as hydrogen bonding between silanol groups and polar analytes can occur, thus facilitating their retention. A recent study has compared recoveries obtained for polar priority phenols using an on-line system, and recoveries were found higher with the monofunctional C_{18}/OH sorbents than standard C_{18} from IST [114]. As examples, using a 100 ml sample and 10×2 mm i.d. precolumns, recoveries were 25% and 33% for 4-methylphenol and 4-nitrophenol with the standard C_{18} and 54% and 56%, respectively, using C_{18}/OH. Stronger secondary interactions can also occur also with basic analytes when both the analyte and the silanol groups are ionised. But, even if retention of polar analytes can be higher with such C_{18} silicas due to secondary interactions, one should realise that a twofold retention induces only an increase of 0.3 units in the log k_w value. The increase in retention using polymeric sorbents is far above, as explained below, and these specific silicas will never compete with the new polymers for extraction of polar analytes.

Capacities of sorbents. One possible cause of breakthrough is the overloading of the capacity of the extraction column or precolumn. Breakthrough curves have been recorded for increasing concentrations of dimethyl phthalate in water on C_{18} [80]. For water spiked with 0.3 and 0.9 ppm, breakthrough occurs at the same percolated volumes but for higher concentrations, the breakthrough volumes decrease and is no longer related to V_r value. Assuming a Langmuir-type adsorption isotherm, overloading occurs when 20 μg of dimethylphthalate are adsorbed on the precolumn which corresponding to about 1 mg/ g of C_{18} silica. The capacity depends on the size of the solute and on its steric configuration. Under similar conditions, it was estimated to 4 mg/g of C_{18} silica for xylene [80]. In the literature capacity values up to 15–60 mg/g of packing material have been reported [35]. Although the total concentration of both solutes and interferences have to be considered, concentration in surface water samples are at the μg/l level so that overloading is rather unlikely to occur.

Desorption conditions. The lower the desorption volume is, the higher the enrichment factor. The elution power decreases within the series hexane, THF, ethyl acetate, methylene chloride, acetone, acetonitrile and methanol. However, most of the medium-polarity analytes are not -or are just slightly retained with pure methanol or acetonitrile, which are often preferred because they are water-miscible. Current volumes are between 2 and 5 ml/ 500 mg of C_{18} sorbent. Ethyl acetate was found to be efficient, and many apolar to moderately polar pesticides were eluted in the first 60 μl of eluate from cartridges containing 100 mg of C_{18} silicas with recoveries higher than 90% [115,116]. The solubility of compounds in the mobile phase plays an important role in reversed-phase chromatography and it is a useful guide for selecting the eluting organic solvent.

When the subsequent analysis is performed by GC, one method consists in eluting the analytes from the C_{18} cartridge with a GC-compatible solvent, after drying it. Another option is the desorption with a water-miscible solvent, evaporation to dryness, and re-dissolution in a GC-compatible solvent. In the first option, some differences in recoveries were observed when pure hexane, and hexane with 15% of methylene chloride, were used for the desorption after percolation of 200 ml of an aqueous sample spiked with organo-chlorine pesticides [117]. The addition of methylene chloride increases the solubility of the analytes and helps to give better contact with the sorbent because traces of waters are still present. Acetone and ethyl acetate are more appropriate solvents for desorption and further GC analysis since the latter forms an azeotrope with water which can be removed during the evaporation to dryness.

Fractionation in a polarity group during the desorption is difficult and is not often reported. A sequential desorption has been described for the determination of alachlor and its major metabolite, ethanesulfonic acid, in water with detection by an immunoassay [118]. Alachlor and its metabolite were isolated from water with a C_{18} sorbent and eluted sequentially with ethyl acetate and methanol because alachlor is very soluble in ethyl acetate while the anionic metabolite is not. Thus the latter remained adsorbed on the C_{18} sorbent and was eluted later with methanol.

Reversed-phase sorbents are often used for preconcentration of ionisable compounds in their molecular forms. Desorption from these sorbents can be performed by a solution adjusted to a pH where the analytes are in their ionic form (two units below or above the pK_a).

Matix effect. The potential for determining many analytes over a wide range of polarity

in drinking water at the low 0.1 μg/l level was shown with the simultaneous determination of triazines and phenylureas [119]. The mixture included some polar analytes such as the degradation products of atrazine, i.e. de-isopropylatrazine, hydroxyatrazine and de-ethylatrazine, and fenuron or metoxuron (with log k_w below or around 2.5), many moderately polar ones and rather apolar pesticides such as neburon (log $K_{ow} = 4.3$). The analytical separation was carried out by reversed-phase chromatography using a C_{18} analytical column and an acetonitrile gradient in phosphate buffer at pH 7. Fig. 1.10 shows the chromatograms at 220 and 244 nm obtained for an extract from 500 ml of drinking
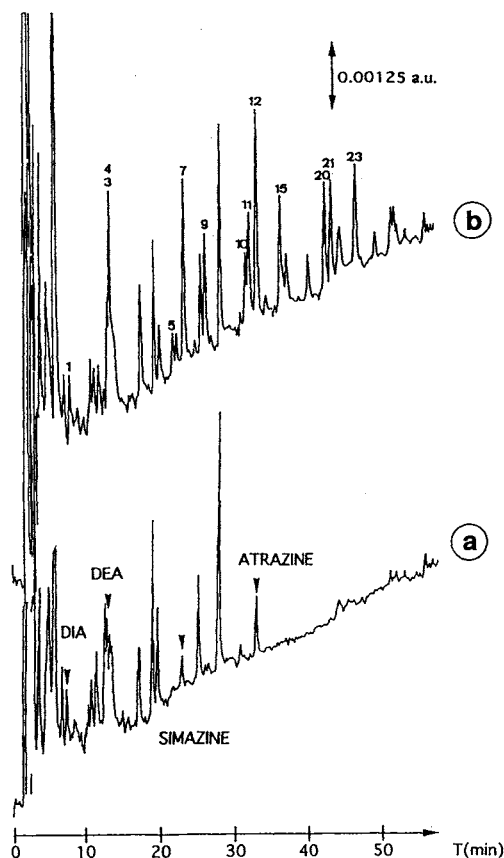
Fig. 1.10. Analysis of an extract from drinking water (a) non-spiked and (b) spiked with 0.1μg/l of each analyte. Preconcentration of 500 ml of drinking water via a 500 mg C_{18} silica cartridge, desorption with 4 ml of methanol, evaporation to dryness, and addition of 500 μl of an acetonitrile/water mixture (20:80, v/v). Injection: 50 μl. Analytical column: Supelcosil LC-18-DB 25 cm × 4.6 mm i.d.; acetonitrile gradient with 0.005 M phosphate buffer at pH 7; UV detection at 220 nm. Peaks: 1, DIA; 2, fenuron; 3, OHA; 4, DEA; 5, hexazinone; 6, metoxuron; 7, simazine; 8, monuron; 9, cyanazine; 10, metabenzthiazuron; 11, simetryne; 12, atrazine; 13, chlortoluron; 14, fluometuron; 15, prometon; 16, monolinuron; 17, isoproturon; 18, diuron; 19, difenoxuron; 20, sebutylazine; 21, propazine; 22, buturon; 23, terbutylazine; 24, linuron; 25, chlorbromuron; 26, chlorooxuron; 27, difluzbenzuron; 28, neburon.

water which was non-spiked (Fig. 1.10a) and spiked (Fig. 1.10b,c) with 0.1 μg/l of each pesticide, after dissolving the dry extract in 500 μl of mobile phase and injecting a 50 μl aliquot into the analytical column. Apart from the early-eluted peaks 1 to 4, for which the recoveries are, respectively 26%, 51%, 68% and 68%, one can see that the detection limits are in the range 0.01–0.05 μg/l. The occurrence of simazine (peak 7) and atrazine (peak 12) was confirmed by comparison of retention times and UV spectra from the library of the DAD at respective concentrations of 0.016 ± 0.003 μg/l and 0.12 ± 0.02 μg/l. The match between the retention times and the two UV spectra was excellent, so no further confirmation is required. The match was no so good for DIA and DEA which were to be confirmed by another method.

No breakthrough should occur for apolar compounds with the handling of at least 1 litre. Another cause of loss in recoveries has been observed, which is not due to breakthrough, but to adsorption of these hydrophobic compounds onto connecting tubes and containers. The adsorption of some non-polar pesticides onto glass and Teflon bottles has been reported. Since this adsorption is low, it is not visible when samples are spiked at the μg/l level or more, but it is when samples were spiked at the 0.25 μg/l level. Recoveries ranged from less than 20% for permethrin, cypermethrin, fenvalerate and DDE, between 30% and 60% for DDD and DDT, above 80% for HCH, dieldrin and endrin, and 100% for atrazine and simazine [120]. Adsorption was in general higher on Teflon than on glass bottles. In order to avoid the adsorption problems, one solution is to add a small proportion of organic solvent (methanol, acetonitrile or isopropanol) to the samples before percolation through the cartridge. Since for apolar compounds the breakthrough volumes are very high, the reduction of breakthrough volume from adding 5–10% of an organic solvent can still allow the handling of 500 ml of sample without breakthrough and consequent loss in recoveries. The extraction of pyrethroid pesticides using C_{18} cartridges packed with 100 mg of sorbent and a sample volume of 27 ml containing 30% methanol was obtained recoveries around 90% for the pyrethroids fenpropathrin, permethrin and deltamethrin [121].

Ionised analytes are usually not, or are only slightly retained by C_{18} silica and the analyte extraction required to adjust the sample pH in order that the analytes are in their uncharged form. In case of moderately acidic compounds (pK_a around 4), the sample should be adjusted at 2 or 3. The recovery of some acidic herbicides with pK_a values in the range 3–5 was around 30% at pH 7 and over 95% at pH 2 when 500 ml of water were percolated through on a 500 mg C_{18} silica cartridge [2]. But when samples are at pH 2, then the co-extraction of humic and fulvic acids occurs in natural waters as shown in Fig. 1.11. The consequence of the humic and fulvic interferences is for the detection limits, which are in the 0.1 μg/l range in drinking water, are closer to 0.5 μg/l in a contaminated surface water. They can be improved provided an additional clean-up step [119].

1.4.3.2 Apolar styrene divinylbenzene copolymer sorbents

The styrene divinylbenzene (SDB) resins of the Amberlyte XAD-type have been widely used in laboratories but were not available in prepacked cartridges because they required laborious purification before use. The first disposable SDB sorbents became available in extraction disks. One advantage over C_{18} silicas is their stability over the whole pH range
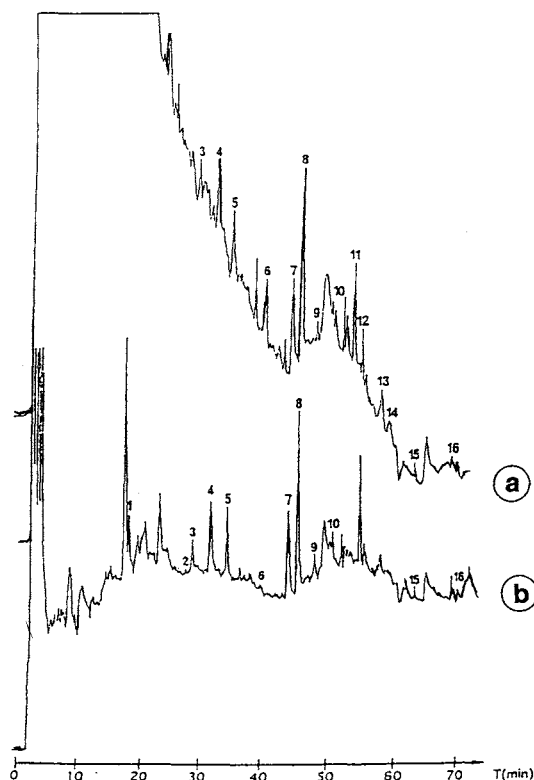
Fig. 1.11. Effect of the matrix sample when the samples are at pH 3 (a) and 7 (b). Injection of an extract from drinking water spiked with 0.1 μg/l of each analyte. Preconcentration of 500 ml via a 500 mg C_{18} silica cartridge, desorption with 3 ml of methanol, evaporation to dryness, and addition of 500 μl of an acetonitrile/water mixture (20/80, v/v). Analytical column: Bakerbond Narrow Pore C_{18} silica, 25 cm × 4.6 mm i.d.; acetonitrile gradient with 0.005 M phosphate buffer at pH 3. UV detection at 220 nm. Peaks: 1, chloridazon; 2, aldicarb; 3, metoxuron; 4, simazine; 5, cyanazine; 6, bentazone; 7, atrazine; 8, carbaryl; 9, isoproturon; 10, difenoxuron; 11, ioxynil; 12, MCPP; 13, 2,4-DB; 14, 2,4,5-TP; 15, metolachlor; 16, dinoterb.

1–14, which was demonstrated when comparing blanks obtained after the percolation of 5 l of water at acidic pH through C_{18} and SDB disks [71].

Four or 5 years ago, the first resins with high specific surface areas, around 1000 m^2/g, became available in disposable cartridges or disk. They are now available for many companies and the commercial data have been reported in Table 1.3. The manufacturers provide recoveries of phenol and deisopropylatrazine for comparison with C_{18} silicas, these recoveries being 100% for sample volume of 1 litre and using only 200 mg of sorbents, showing thus a much higher retention than C_{18} silica.

Prediction of k_w. Because these polymers are not available in analytical columns (they do not possess all the properties required), very few chromatographic data have been reported up to now. LC data are available only for LC-grade SDB with specific surface area of 415 and 550 m^2/g (PRP-1 and PLRP-S, respectively). Retention behaviour of

TABLE 1.3

CHARACTERISTICS OF COMMERCIALLY AVAILABLE APOLAR COPOLYMERS USED AS LC AND SPE SORBENTS

Sorbent	Manufacturer	Porosity (A)[a]	Average dp (μm)	Surface area (m^2/g)
Bond-Elut ENV	Varian	450	125	500
Bond-Elut PPL	Varian	300	125	700
SDB	J.T. Baker	300	40–120	1060
Speedisk-DVB	J.T. Baker	150	n.a.	700
Empore disk	J.T. Baker	n.a.	6.8	350
Lichrolut EN	Merck	80	40–120	1200
Isolute ENV+	IST	100	90	1000
Envichrom P	Supelco	140	80–160	900
Chromabond HR-P	Mach. Nagel	n.a.	50–100	1200
Porapak RDX	Waters	55	120	550
OASIS HLB	Waters	55	30 and 60	800
PRP-1	Hamilton	75	5 and 10	415
PLRPS	Polymer Lab	100	15 and 60	550
Hysphere-1	Spark Holland	n.a.	5–20	>1000

[a] n.a., not available in data supplied by manufacturers.

analytes on PRP-1 sorbent has been studied and compared to retention obtained with C_{18} silicas. First, it was shown that log k_w could be also extrapolated from the relation log k_w-methanol content, as was shown in Fig. 1.8 [2,8,93].

Relation between k_w and the water-octanol partition coefficient. The retention behaviour of analytes is governed by hydrophobic interactions similar to those with C_{18} silicas, but, owing to the aromatic rings in the network of the polymer matrix, one can expect strong electron–donor interactions (π–π) with aromatic rings of solutes. For a set of many organic compounds, the results indicated in Fig. 1.9 show that solutes are about 10 to 40 times higher retained by PRP-1 than by C_{18} silicas. However, the relationship between extrapolated log k_w values and log K_{ow} values is less linear than that existing with C_{18} silicas. The highest difference was for benzene derivatives substituted by nitro groups having a strong electron-withdrawing effect and the smallest for hydroxy group showing an electron-donating effect. The slope of the curves are not the same for C_{18} silicas and for PRP-1. The difference is higher for hydrophobic compounds that for polar ones. For log K_{ow} values below 1, the difference in retention between C_{18} silica and PRP-1 is no longer observed.

Effect of the surface area on retention. The effect of the specific surface area is important as shown in Table 1.4. In order to estimate log k_w values in water-rich mobile phases, a 5-cm and a 3-cm long columns were, respectively laboratory-packed with one of those high specific area SDB (here named HSA/SDB) polymer and with a stacking of SDB polymer disks [94]. Data on C_{18} silica has also been reported for comparison. The retention factors are similar for PRP-1 and SDB disk, but the specific surface area are not very different (415 and 350 m^2/g, respectively) and are higher that those observed with C_{18} silica. With HSA/SDB, there is a large increase in retention, since the difference is

TABLE 1.4

COMPARISON OF log k_w VALUES OBTAINED WITH C_{18} SILICAS, VARIOUS SDB COPOLYMERS (WITH DIFFERENT SPECIFIC SURFACE AREAS, IN m^2/g) AND POROUS GRAPHITIC CARBONS

Compounds[a]	log K_{ow}	log k_w[b]				
		C_{18}	PRP-1 (415)	SDB (disk) (350)	HAS/SDB (1060)	PGC
Cyanuric acid	−0.2	<0.5	<0.5	nd	<0.5	2.6 ± 0.1
Ammeline	−1.2	<0.5	<0.5	nd	<0.5	2.4 ± 0.2
Ammelide	−0.7	<0.5	<0.5	nd	<0.5	2.5 ± 0.2
Hydroxy-DIA	−0.1	1.0 ± 0.1	1.0 ± 0.1	nd	1.8 ± 0.1	3.0 ± 0.2
Hydroxy-DEA	0.2	1.5 ± 0.1	1.8 ± 0.1	nd	2.3 ± 0.2	2.8 ± 0.2
DEDIA	0	1.3 ± 0.1	1.2 ± 0.1	nd	nd	2.8 ± 0.1
Deisopropylatrazine (DIA)	1.2	2.3 ± 0.1	3.1 ± 0.1	3.2 ± 0.2	4.4 ± 0.3	>3.5
Deethylatrazine(DEA)	1.4	2.7 ± 0.1	3.5 ± 0.3	3.5 ± 0.2	4.8 ± 0.3	3.2 ± 0.2
Simazine	2.3	3.4 ± 0.1	> 4	4.1 ± 0.2	5.9 ± 0.3	>4
2-Chlorophenol	2.4	2.9 ± 0.1	>4	3.6 ± 0.2		>4
Oxamyl		1.7 ± 0.1	nd	2.8 ± 0.2	4.1 ± 0.3	nd
Aldicarb	1.4	2.5 ± 0.1	nd	4 ± 0.2	5.3 ± 0.3	nd
Carbendazim	1.5		nd	nd	5.7 ± 0.3	>4
Chloridazon		2.3 ± 0.1	nd	3.8 ± 0.2		>4

[a] Cyanuric acid: 2,4,6-trihydroxy-1,3,5-triazine; Ammeline: 2,4- diamino-6-hydroxy-1,3,5-triazine; Ammelide: 2-amino-4,6-dihydroxy-1,3,5-triazine.

[b] log k_w values extrapolated from the relationships log k-percentage of methanol.

between 1.3 and 1.8 in log unit, indicating that this polymer has 20–60-fold more retention power towards polar pesticides than have polymers with lower specific areas. Comparison with C_{18} silica indicate retention data higher than 100–200-fold. Similar values of k_w for deisopropylatrazine and deethylatrazine have been extrapolated using SDB, EnviChom P and Isolute ENV+. The retention order is similar to that obtained with C_{18} silica and the higher the hydrophobicity of the molecule is, the higher retention. But, there is a limit in polarity for extraction of compound. In Table 1.4, one can see that log k_w values are lower than 2 for the highly polar degradation products of atrazine.

These HSA/SDB are the sorbents to be selected for the extraction of very polar analytes when large sample volumes are required [2,6,21,96,114,122–131]. Table 1.5 shows examples of high recoveries obtained from 1 litre samples. A study also reported excellent recoveries for the extraction of some polar organophosphorus pesticides using LiChrolut EN or SDB [124].

Slight sulfonation of SDB resins was shown to provide a better contact with aqueous samples and to increase the retention of polar analytes [132,133]. More recently, the high capacity resins have been chemically modified by various hydrophilic groups such as acetyl or carboxybenzoyl groups and higher recoveries were obtained for polar phenolic compounds [134–136].

Matrix effect: removal of the humic and fulvic interferences. Recent works have also shown that ionic organic compounds are well retained by these HSA/SDB owing to interactions between the SDB matrix and the organic part of the compounds [94]. This is of high interest for the analysis of acidic analytes (ionisation constants in the range 3–6) which can be extracted under their ionic form from surface waters at pH 7–8 with good recoveries using 500 ml samples. Using C_{18} silicas, the extraction of acidic compounds required the acidification of the samples in order to have these acids in their neutral form, because low recoveries are obtained for ionic compounds. But, then, most of the polar compounds cannot be determined due to a large matrix peak obtained at the beginning of the chromatogram when surface water samples are analysed. Therefore, polar analytes can be determined at trace level samples can be analysed at pH 7 because there is a clear baseline at the beginning of the chromatogram as shown in Fig. 1.12. This figure highlights the interest of handling the samples at pH 7, since it is possible to detect analytes at the 0.1 μg/

TABLE 1.5

RECOVERIES (%) OF EXTRACTION OBTAINED FOR POLAR PESTICIDES IN WATER SAMPLES SPIKED AT 0.1 μg/l ON 47 mm C_{18} DISK (450 mg OF SORBENT, J.T. BAKER, SAMPLE 500 ml), 47 mm SDB DISK (SDB, J.T. BAKER, 450 mg OF SORBENT, SAMPLE 1 litre) AND ON A 200 mg HSA/SDB CARTRIDGE (J.T. BAKER, SAMPLE 1 l)

Solute	log K_{ow}	C_{18} Disk	SDB Disk (350)	HSA/SDB cartridge (1060)
Oxamyl	−0.47	<3	27	82
Deisopropylatrazine	1.1	21	53	92
Deethylatrazine	1.5	58	93	100
Carbendazim	1.56	62	84	88
Aldicarb	1.1–1.5	69	72	90
Simazine	1.96	95	90	94
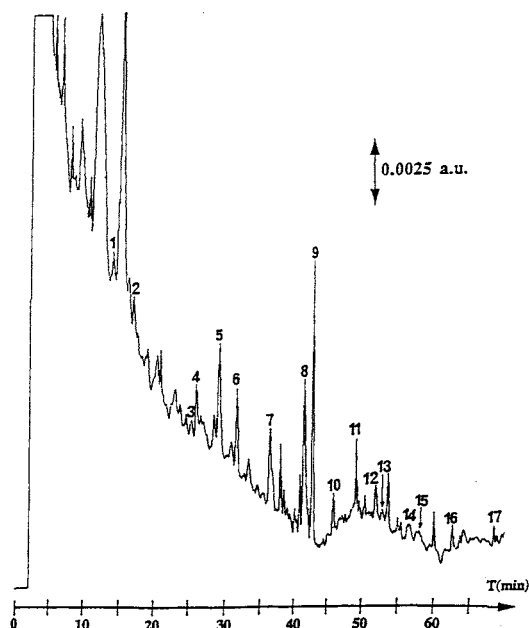
Fig. 1.12. Effect of the pH of the sample and of the matrix of the sample. Analysis of an extract from 500 ml of River Seine water spiked with 0.1 μg/l of herbicides. Preconcentration through a 200-mg SDB cartridge, desorption with 4 ml of methanol, evaporation to dryness, and addition of 200 μl of an acetonitrile/water mixture (20:80, v/v). Analytical column: Bakerbond Narrow Pore C_{18} silica, 25 cm × 4.6 mm i.d.; acetonitrile gradient with 0.005 M phosphate buffer at pH 3. UV detection at 220 nm. Peaks: 1, chloridazon; 2, dicamba 3, aldicarb; 4, metoxuron; 5, simazine; 6 cyanazine; 7, bentazone; 8, atrazine; 9, carbaryl; 10, isoproturon; 11, ioxynil; 12, MCPP; 13, 2,4-DB; 14, 2,4,5 TP; 15, metolachlor; 16, metolachlor; 17, dinoterb.

l level in contaminated surface water (River Seine sampled in Paris) in a single extraction/ preconcentration step.

Desorption conditions. As a result of the primary hydrophobic retention mechanism, compounds are not, or are only slightly, retained by organic solvents, and the same eluotropic series as described for C_{18} silicas can be observed. However, compounds being more retained on SDB sorbents than on C_{18} silica, a higher volume than twice or three times the void volume of the cartridge will be required. This should be considered when non-polar analytes are to be determined. There is an interest in analysing moderately to non-polar analytes with the addition of 10% of an organic solvent for handling waste water of contaminated surface water. Advantages are to avoid eventual by adsorption of hydrophobic analytes and to remove many polar interferences, usually seen as a peak at the beginning of chromatograms. Since the analytes are strongly retained desorption is more efficient when using a mixture of methanol and methylene chloride [21,123].

1.4.3.3 Carbon-based sorbents

Carbon-based sorbents are more and more used for the extraction of polar compounds

and several carbon-based sorbents are now available for SPE in water [137]. The most widely used carbon-based SPE are graphitised carbon blacks (GCB) obtained by heating carbon blacks at high temperature (2700–3000°C). The first GCBs were non-porous with a low specific surface area (Carbopack B or ENVI-Carb SPE from Supelco, Carbograph from Altech). Their higher efficiency over C_{18} silica for trapping polar pesticides have been extensively shown by the group of Di Corcia et al. [20,138–143]. This is illustrated in Table 1.6 with the comparison of recoveries for some polar compounds obtained with a cartridge packed with 500 mg of C_{18} silica and another one packed with 250 mg of Carbopack when 2 l of spiked water are handled. Carbograph 4 was introduced with a surface area of 210 m^2/g [143]. As all carbonaceous sorbent made from carbon blacks, various functional groups are present at the surface following the oxygen chemisorption. Taking advantage of the positively charged active centres at the GCB surface multiresidue methods for pesticide analysis gave been performed which involved a fractionation between neutral and basic pesticides on one hand and acidic in another hand [140,144–146]. The determination of 15 post-emergence herbicides were obtained with detection limits of 5 ng/l from the preconcentration of 4 l of drinking water using a reversible extraction cartridge packed with 0.5 g of Carbograph 4 [147]. This property was also exploited for the extraction of benzene and naphthalene sulfonate and was shown to be more efficient that conventional ion-pair extraction on C_{18} silicas [148]. Carbograph 4 was used for the extraction and identification using LC-MS of biotransformation products of alcohol ethoxylate surfactants [149,150]. Carbon-based membrane extraction disks are also available and were used for the determination of N-nitrosodimethylamine at the ng/l level in ground water [151].

Prediction of retention data from retention mechanism. Graphitised carbon blacks are not enough pressure resistant to be used in liquid chromatography so that no data indicating the LC behaviour of solutes are available. Porous graphitic carbon (PGC) is available in SPE cartridges (Hypersep PGC) and is similar to the LC-grade Hypercarb, which appeared at the end of the 1980s [152]. It is characterised by a highly homogeneous

TABLE 1.6

COMPARISON OF RECOVERIES (%) OBTAINED WITH (A) CARTRIDGES CONTAINING 500 mg OF C_{18} SILICA FROM SUPELCO AND (B) CARTRIDGES CONTAINING 250 mg OF GRAPHITISED CARBON BLACK, CARBOPACK FROM SUPELCO; SAMPLE VOLUME OF 2 l SPIKED WITH 0.25–1.5 μg/l OF EACH PESTICIDE

Solute	C_{18}	Carbon
Oxamyl	4	89
Methomyl	3.7	98
Chloridazon	18	98
Metoxuron	64	97
Bromacyl	53	96
Monuron	49	100
Carbofuran	64	98
Carbaryl	78	96
Bromoxynil	33	96
2,4 D	41	93

and ordered structure and by a specific area around 120 m^2/g. Fig. 1.8 has shown the reversed-phase behaviour and that the retention factor log k_w can be extrapolated from the same relation as that observed with C_{18} silicas or SDB polymers. However, the retention mechanism was shown to be very different from that observed on C_{18} silicas or SDB polymers and due to its crystalline structure made of large graphitic sheets held together by weak Van der Waals forces [153]. Both hydrophobic and electronic interactions are involved in the retention mechanism, so that non-polar analytes, but also very polar and water-soluble analytes were shown to be retained in water [93,153–160]. Therefore, log k_w cannot be predicted easily and there is no relation between log k_w and log K_{ow} except for a series of related analytes such as alkylbenzenes. There is even no link at all between the retention order and the hydrophobicity and polarity of the molecule. The affinity of PGC towards very polar and water-soluble polyhydroxybenzenes has been studied [154]. The capacity factor in water of the very polar 1,3,5-trihydroxybenzene (phloroglucinol) was about 1000 with PGC whereas it was found three (log k_w of 0.5) with PRP-1. This compound is not retained by C_{18} silica and it was even proposed as an experimental probe for determining the void volume of C_{18} columns. Other extrapolated or real log k_w values have been measured for mono- and polysubstituted benzene derivatives with RP-18, PRP-1 and with PGC Results are reported in Table 1.7. First, when comparing values for monosubstituted benzenes, compounds are more retained by PRP-1 than they are by PGC. The comparison between RP-18 and PGC indicates that solutes are less or more retained by PGC than they are by RP-18. In contrast to results on PRP-1 indicating that retention of all the solutes were higher with PRP-1 than that with C_{18} silicas, no correlation was found between retention of monosubstituted benzenes on PGC and retention on C_{18} silicas. The disubstituted benzenes studied in Table 1.7 are rather polar compounds and are not, or slightly, retained by C_{18} silicas, explaining why log k_w values have not been

TABLE 1.7

COMPARISON OF EXTRAPOLATED log k_w VALUES OBTAINED WITH RP-18 SILICA, PRP-1 AND PGC

Solute	RP-18	PRP-1	PGC
Monosubstituted			
Benzene	2.2	3.5	1.45
Aniline	1.08	2.5	1.35
Phenol	1.55	2.4	1.8
Benzoic acid	1.9	3.2	2.4
Nitrobenzene	2.05	3.6	2.45
Polysubstituted			
4-Aminophenol		1.1	2.05
1,4-Diaminobenzene		1.2	2.4
4-Aminobenzoic acid		2	2.85
4-Hydroxybenzoic acid		2.3	2.7
3,5-dihydroxybenzoic acid		1.35	3
1,3-Dihydroxybenzene		1.35	2.35
1,4-Dihydroxybenzene		0.83	2.15
1,3,5-Trihydroxyphenol		0.5	2.7

reported. The comparison between the retention obtained on PRP-1 and on PGC are interesting. With PRP-1 log k_w obtained with two polar substituents are always lower than that measured for each corresponding monosubstituted benzene whereas the contrary is observed with PGC. For instance, log k_w of aminophenol is 1.1 with PRP-1 and is lower than both log k_w of phenol (2.4) and aniline (2.5). With PGC, log k_w of aminophenol is 2.05 and is higher than log k_w of both phenol (1.8) and aniline (1.35). The retention mechanism is therefore very different for the two sorbents.

High retention are usually obtained for planar molecules containing several polar groups with delocalised electronic charges via π-bonds and lone pairs of electrons. The potential of PGC for extracting very polar compounds was also demonstrated in Table 1.4 for dealkylated and hydroxylated degradation products of atrazine down to cyanuric acid whereas the limitation of both C_{18} silica and polymer are clearly shown for the very polar ammeline, ammelide and cyanuric acid with log k_w values lower than 0.5 whereas they are higher than 2 with PGC. Using a 200 mg PGC cartridge, recoveries were above 90% with the handling of 250 ml of water sample for all the metabolites except the three more polar ones for which a 500 mg cartridge was required to obtained similar recoveries [95]. Fig. 1.13 shows the chromatogram of an extract from 300 ml of drinking water spiked with some polar metabolites of atrazine. This chromatograms shows the different retention mechanism of the carbon used both for the extraction and for the analysis. The retention order between DEA and DIA is the inverse of that observed using a C_{18} analytical column.

Di Corcia et al. have described the ultratrace determination of atrazine and its six major
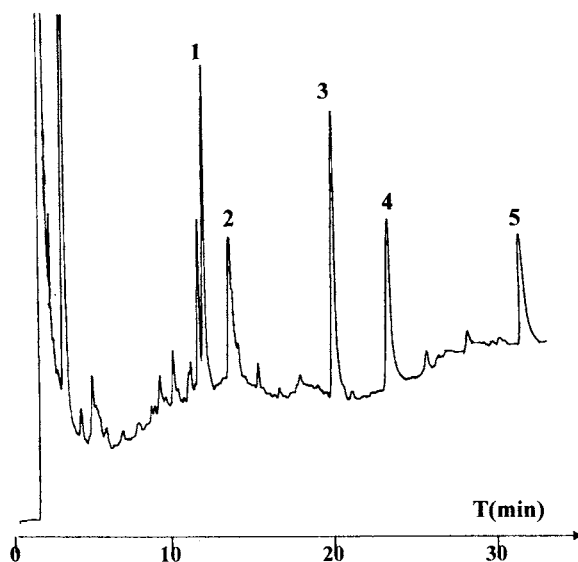
Fig. 1.13. Analysis of an extract from 500 ml of drinking water spiked with 0.3 μg/l of each analyte. Preconcentration on a 200 mg cartridge packed with Hypercarb. Analysis using a Hypercarb analytical column (100 × 4.6 mm), acetonitrile gradient with 0.005 M phosphate buffer at pH 7 from 10% to 70% acetonitrile from 10 to 35 min. UV detection at 220 nm. Peaks: 1, De-ethyl,de-isoprolyla-trazine; 2, hydroxyatrazine; 3, de-ethylatrazine, 4, de-isopropylatrazine, 5, atrazine.

degradation products using SPE with Carbograph 4 followed by LC and electrospray MS [161]. When polar phenols only included chlorophenols and higher chlorinated ones, comparison between GCB and highly cross-linked polymer gave similar results [162].

Since no guide can be given for log k_w prediction, the only rapid and easy mean is to inject the polar analyte of interest onto an available analytical column of PGC with a methanol-water mobile phase and to estimate log k_w values via the relation log k-methanol content.

Desorption conditions. Problems of elution have been pointed out [163–165]. Owing to the different retention mechanism, acetonitrile and methanol can be inefficient and it is preferable to use methylene chloride or THF. This was demonstrated by LC measurements showing that several analytes are still strongly retained with pure methanol or acetonitrile as mobile phase [164]. Therefore, when a multiresidue extraction is performed, it is highly recommended to allow desorption in the backflush way compared to the percolation way, because it is impossible to predict which compound will be retained or not by pure organic solvent. Cartridge allowing percolation and desorption in the opposite way are now available.

1.4.3.4 Ion-exchange sorbents

Ionic or ionisable analytes can be extracted by ion-exchange sorbents. Most of the disposable cartridges are prepacked with silica-based sorbents which have the inherent disadvantages over polymers of being limited to the pH range 3–9 and having a lower capacity. Cation-exchanger includes weak carboxylic acid and strong aromatic or non-aromatic sulfonic acid groups. Weak anion-exchanger groups are made of primary or secondary amino groups whereas strong anion exchangers are quaternary amine forms. They are also available in precolumns and disks. The method development is easy for ionisable analytes because retention occurs for a sample pH allowing the analyte to be in its ionic form whereas desorption in its neutral form. If the analytes are ionic over the whole pH range, then desorption occurs by using a solution of appropriate ionic strength, according to the basic principles of ion-exchange chromatography.

The main problem encountered when environmental are handled comes from the fact that they contain high amounts of inorganic ions which overload the capacity of these sorbents. A chemical sample pretreatment based on precipitation of calcium with oxalic acid and complexation of iron with EDTA can been carried out in an on-line procedure [166]. The method was applied to the preconcentration of the pesticide aminotriazole which is polar and water-soluble, and not retained on C_{18} silica or polymers [167,168]. The breakthrough volume on a precolumn (10×2 mm i.d.) prepacked with a sulfonic acid-type of resin-based cation-exchanger was measured as 150 $\pm$ 10 ml with LC-grade water spiked with aminotriazole. With drinking water samples, the breakthrough volume was below 5 ml. After the chemical pretreatment to remove inorganic anions, the recovery with a 30 ml sample of spiked drinking water was 18% as a result of the competition between the remaining trace inorganic ions and organic ions, in favour of the inorganic ions. When the organic ions of interest are more hydrophobic, then additional interactions occur with the matrix of the ion-exchanger sorbent, so that the competition is in favour of the organic ions. One example is in the direct concentration of triazines at low pH using cation-exchanger cartridges.

Few applications have been described using anion exchangers. Trifluoroacetic acid was quantitatively recovered from most environmental waters by an extraction procedure using an anion-exchange Empore disk [169]. Using the same type of disks, another study described the extraction of the negatively charged pesticide dacthal and its metabolites in ground water [170,171]. A two step procedure coupling a first cartridge of Lichrolut and then a second one packed with a strong anion exchange (SAX) was used for the extraction of glyphosate and its main metabolite aminophosphoric acid from water [172].

1.4.3.5 Metal-loaded sorbents

Organic compounds which can form complexes with metal ions can be preconcentrated selectively by metal-loaded sorbents. A silica containing the functional group 2-amino-1-cyclopentene-1-dithiocarboxylic acid (ACTA) loaded platinum(IV) irreversibly retained aniline from water [173]. This sorbent was used to remove interfering anilines in the determination of phenylurea herbicides. The mercury-8 hydroxyquinoline phase allowed the preconcentration of 2-mercaptobenzimidazole [174] whereas Ag(I) oxine was preferred for the determination of buturon in water [175].

Preconcentration on silicas modified with complexation properties has been reviewed by Veuthey et al. [176]. Some applications of on-line preconcentrations with metal-loaded precolumns have been reported by Nielen et al. [78].

1.4.3.6 Immunoextraction sorbents

The wide range of the SPE sorbents described up to now are non-selective, -except ion-exchangers. Consequently co-extraction of analytes and interferences generally occurs with the handling of dirty samples or complex samples and sometimes analytes of interest are at trace-level and interferences at higher concentrations. As an example, most of the polar organic compounds cannot be determined at trace-level by LC due to their co-elution with humic and fulvic substances present in high amount in soil and natural waters. Evidence of these compounds are usually seen as an important interfering matrix peak at the beginning of the chromatogram and additional clean-up procedures are usually required prior to the final chromatographic analysis.

Immunoextraction sorbents (ISs) are obtained by bonding antibodies onto a sorbent, and their main feature is their high selectivity resulting from the antigen–antibody interactions. Since antibodies are highly selective towards the analyte used to initiate the immune response with a high affinity, the corresponding immunosorbent may extract and isolate this analyte from complex matrices in a single step, and the problem of the co-extraction of matrix interferences is therefore circumvented.

The first ISs have been described in the biological field because of the availability of antibodies which can be very selective for large molecules. In the environmental field, immunoaffinity cartridges are available for the clean-up of food extracts for the determination of mycotoxins [177–183]. The binding of analyte to antibody is the result of a good spatial complementary and is a function of the sum of intermolecular interactions. Therefore, an antibody can also bind one or more analytes with a structure similar to the analyte which has induced the immune response, and this is the so-called cross-reactivity of

antibodies. It is usually a negative feature for immunoassay, but it was exploited in extraction. Sepharose- or silica-based ISs are now used for preparing immunoextraction sorbents because they do not give raise to non-selective interactions and extraction can occur only by the selective immunoaffinity interactions. The advantage of silica is its pressure resistance so that it can be used directly in on-line set-up in a precolumn. Aldehyde activated silica was used for bonding antibodies anti-carbofuran and demonstrated excellent specificity toward this single analyte with direct extraction and detection at low levels (40 ng/l) in spiked water [184,185]. The selectivity was shown with the analysis of a crude potato extract. Other studies were published targeting pesticides such as atrazine and terbutylazine [186], atrazine and its major metabolites [186], chlortoluron [187], isoproturon [188] or carbendazim [189]. The cross-reactivity of antibodies was also exploited for developing ISs that could selectively extract a whole class of structurally related compounds. ISs have been tailored by several authors for the extraction of groups of organic compounds including triazine and phenylurea pesticides, BTEXx (benzene, toluene, ethylbenzene and xylene isomers), polyaromatic hydrocarbons (PAHs), benzidine and related azo dyes [190–200]. In order to recover the whole class, sometimes two antibodies have been mixed in the cartridge bed [198].

The main properties of these new extraction sorbents have been described in recent reviews [199,200]. Cartridges packed with silica-based IS are as easy to use as C_{18} silica cartridges, with activation, percolation of the samples, and desorption with a few ml of methanol-water mixture (70:30, v/v). A convincing illustration of the high selectivity provided by IS is shown in Fig. 1.14 which shows the chromatograms corresponding to the extraction of 50 ml of dirty surface water non-spiked and spiked with 0.1 μg/l of a mixture of triazines through a cartridge containing 0.5 g of IS anti-atrazine [192]. The drastic reduction of interferences by matrix constituents allows a more reliable identification of pesticides at very low detection levels. The selectivity of the preconcentration is so high that the sample volume could be reduced to 50 ml. Detection limits were in the 0.03 μg/l range for these triazines in surface water. Similar results have been obtained for phenylureas [201].

1.4.3.7 Molecular imprinted polymers

The high selectivity provided by immunoextraction has led to attempt to synthesise antibody mimics. One approach has been the development of molecularly imprinted polymers (MIPs) these recent years. They involve the preparation of polymers with specific recognition sites for certain molecules. The synthesis is made by assembly of monomers around a template molecule and a subsequent polymerisation using a cross-linker providing thus a rigid material. Then, the template molecules are removed and the resulting polymers have cavities which are the 'imprints'. These cavities are the recognition sites allowing binding of the template molecule. Like immunosorbents, the recognition is due to shape and a mixture of hydrogen, hydrophobic and electronic interactions. However, they have the advantages to be prepared more rapidly and easily, using well defined methods, and to be stable at high temperature, in a large pH range and in organic solvents. MIPs have found applications in liquid chromatography as normal and chiral stationary phases [202,203] and in areas where they can be substitutes of natural anti-
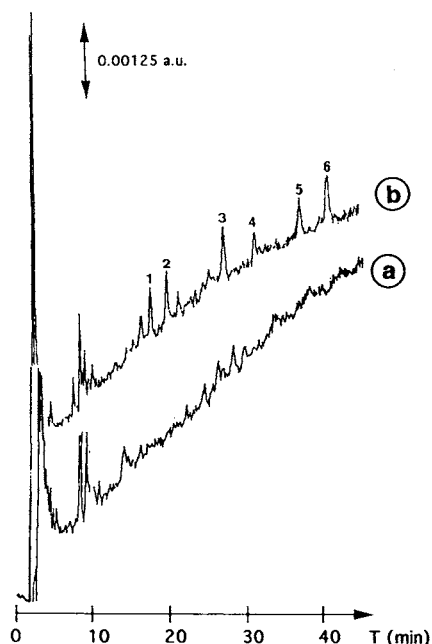
Fig. 1.14. Analysis of a 50 ml surface-water sample extract (a) non-spiked and (b) spiked with 0.1 μg/l of a mixture of triazines. Extraction through a 0.5 g cartridge packed with an immunosorbent anti-triazine IS. Reversed-phase LC with water-acetontrile gradient. Solutes: (1) simazine, (2) cyanazine, (3) atrazine, (4) prometon, (5) propazine, (6) terbutylazine.

bodies, i.e. immunoassays and sensors and solid-phase extraction [204–209]. MIPs are today a challenge as seen by several recent reviews [210–213].

However, as far as now, MIPs for SPE have been optimised to work in organic solvents. So, they are used as clean-up of organic extracts. Few applications have been described in the environmental field. One relevant example is the clean-up of beef liver extracts for the determination of atrazine [207].

1.4.4 Advantages and practical problems

In this section, emphasis has been given to the theoretical basis of SPE, in order to be able to select both sorbent, and the sample volume, the key-parameters of this method. The different sorbents have been discussed, with regards to their ability of trapping a wide range of analytes with different polarities or their selectivity. New SPE sorbents are providing better wettability and emerging ones are based on molecular recognition.

It is always a challenge to extract as much as possible analytes in one run in order to decrease the price and the time of the analysis in the environmental field. However, the probability is high to have in the mixture analytes with different polarities, water solubility, ionisation properties and volatility. But, as far as high cross-linked SDB sorbents have now the capability of trapping both polar, non-polar and ionised organic analytes, the challenge may be now possible and is very attractive. However, one must be aware of

simple practical problems coming from the physico-chemistry properties. A first one occurs during the sample percolation, because recoveries of hydrophobic analytes with very low water solubility are low unless a certain percentage of organic solvent is added in the sample. But if the addition of an organic solvent solves the problem of the hydrophobic ones, it decreases the breakthrough volumes of the more polar ones. Another problem is in the reconstitution of the extract. When very polar and non-polar analytes are together, complete solubilisation of the extracts is often impossible: addition of water is required for the more polar ones, whereas very hydrophobic analytes can only be dissolved a non-polar organic solvent. Therefore, the range of polarity and water solubility should be carefully checked for performing a good multiresidue analysis. Sometimes, it is more rapid to split the list of analytes to be determined in two, polar and moderately polar on one hand and non-polar with the addition of organic solvent (providing also some degree of clean up) in another hand.

Nevertheless, advantages are more numerous in comparison with LLE:

- simplicity;
- speed and possibility of predicting the experimental parameters (sample, volume, sorbents);
- sampling in the field, avoiding transport of voluminous samples, and allowing a good storage;
- efficiency: no emulsion, purer samples;
- safety: use and disposal of flammable solvent and exposure of chemists to toxic solvents are reduced to a large extent;
- low cost: less labour, solvent and transport;
- easy automation and possibility of on-line coupling with the separation step.

1.5 CLEAN-UP OF SAMPLES

The clean-up is an important step for determination of organic compounds at low levels and depends of course on the complexity of the matrix sample and of the detection mode especially when the analysis is performing with LC. It is less important when carrying out postcolumn reaction or using selective detection mode such as fluorescence. In most cases, it is not necessary for ground and drinking water. For more complex samples, such as surface, run-off, waste or soil water samples, selective extraction offers an elegant solution since in one step, analytes are extracted and concentrated without a requirement for further clean-up. However, most of the current methods are non-selective LLE or SPE, which yield an extract that often contains too many interfering analytes for easy analysis without clean-up.

1.5.1 Clean-up of total extracts

Widely used clean-up of extracts are based on fractionation of the extracts by LC. A typical scheme of this procedure is shown in Fig. 1.15. Silica, Alumina or Florisil (synthetic magnesium silicate), packed in cartridges or glass columns, are widely employed for fractionating the extract. Step-elution with solvents of increasing polarity allows a separation into fractions on the basis of polarity differences. Such a procedure was
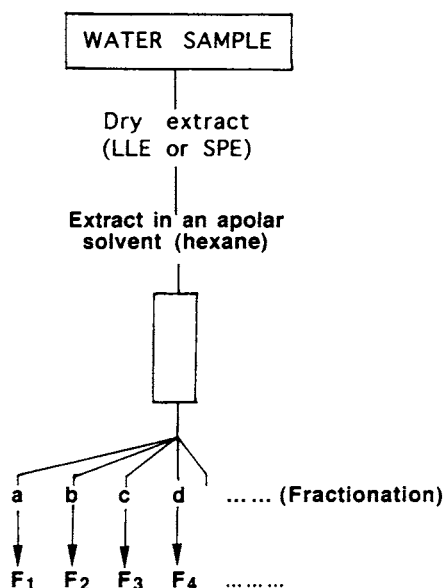
Fig. 1.15. Typical scheme for the fractionation or clean-up of an extract. After injection of the extract, fractionation occurs by eluting the column with eluents a, b, c, d, etc., of increasing polarity

employed by Valls et al. [214], for the determination of ionic and non-ionic contaminants in urban waste and coastal waters. The fraction F1 was eluted by hexane and contained aromatic hydrocarbons; by adding increasing percentages of methylene chloride, methanol and diethyl ether in the eluting mixture, they could obtain seven fractions containing linear alkylbenzenes and polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs) and waxes, fatty acid methyl esters, alkyl and aryl phosphates and ketones, sterols, and, in the last eluted fraction, nonylphenol polyethoxylates. Each fraction is then evaporated, and often derivatised prior or GC–mass spectrometry (MS) analysis. This analytical procedure contains so many steps that it is very time-consuming and unsuitable for automation. The only advantage is its broad screening for the identification of unknown compounds. It is not well adapted to the rapid determination of target compounds but, as it has long been the recommended EPA method for the determination of many priority pollutants, it is still widely used with an optimisation of the fractionation between interferents and analytes.

A more rapid semi-preparative separation of lipid extracts from aquatic media was proposed by liquid chromatography on a silica column [215]. The saponified extract was directly injected on the column and then eluted by a mobile phase of isooctane containing from 0.5% to 10% of 2-propanol. In a single injection the following classes could be separated with good resolution: alkane, aromatic hydrocarbons, fatty acids, alcohols, sterols and hydroxy-fatty acids, according to Fig. 1.16.

Clean-up of organochlorine and pyrethroid insecticides [43] has been performed with an automatic unit, the ASPEC (Automatic Sample Preparation with Extraction Columns from Gilson). The extract has been obtained by LLE from 15 ml of surface water with hexane evaporated down to 1 ml. Clean-up is made with a 100 mg silica cartridge and the whole
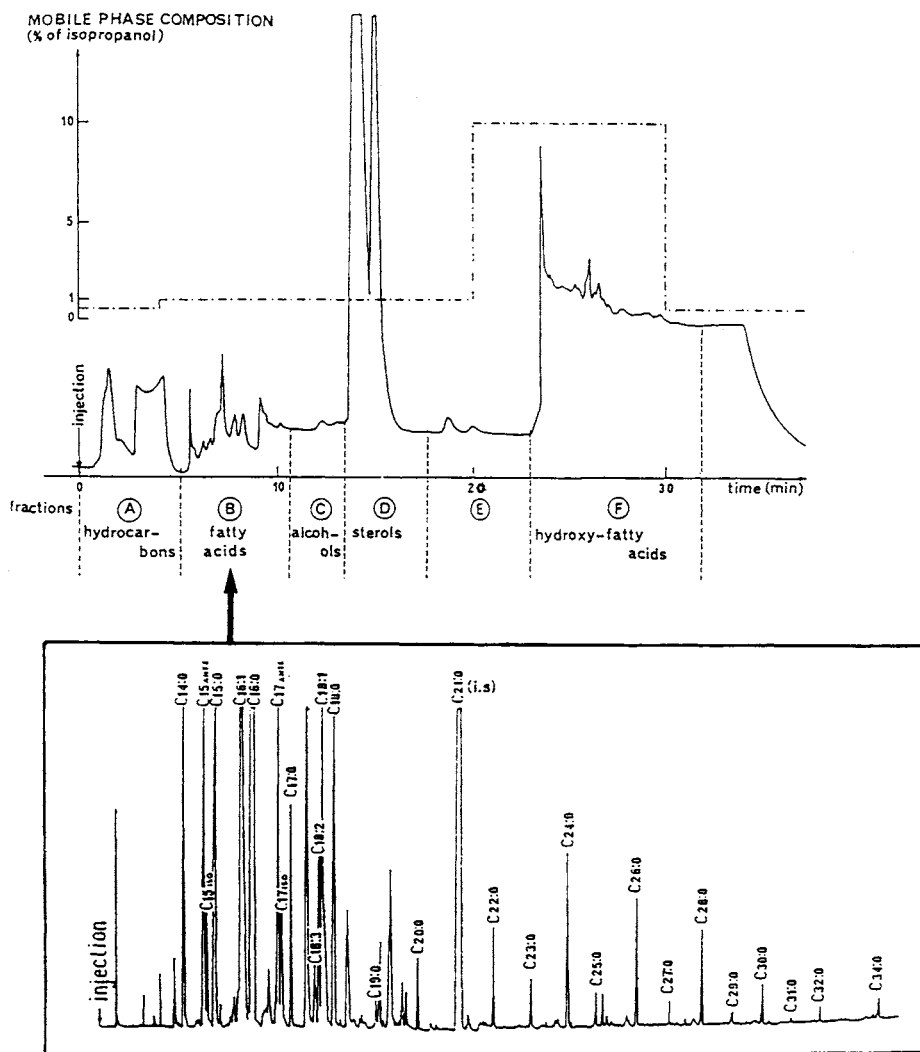
Fig. 1.16. (a) Fractionation of a lipidic extract using rapid semi-preparative liquid chromatography from a standard solution after dissolution of dry extract in isooctane with 0.5% of isopropanol and injection of 1.115 ml. (b) Gas chromatogram corresponding to the fraction B obtained with a natural extract after derivatisation. From [215].

sequence (conditioning, sample washing, eluting) is performed by the ASPEC, which has been coupled on-line to capillary GC-ECD by means of a loop-interface equipped with a solvent vapour exit. The complete analytical procedure is greatly facilitated by automation and considerable decrease in the sample volume required with determination of synthetic pyrethroids at ppt levels in surface water.

The selectivity is the most important feature of immunoextraction sorbents and has been employed for the clean-up of extracts from complex solid matrices such as soil, sediments,

sludges, plant tissue and food. For these samples, there is a real interest in having rapid methods for extracting as much as possible the analytes from solid matrices and then applying immunoclean-up to the extract. SFE coupled to immunoextraction clean-up has been investigated for the trace analysis of organic pollutants including PAHs and pesticides from soil and soots [216]. A nice illustration of the high selectivity provided by immunoextraction is shown in Fig. 1.17 with the comparison of two chromatograms corresponding to the analysis of the same soil extract. One (Fig. 1.17a) is obtained after a classical solvent extraction, dilution of the extract in water and then on-line analysed
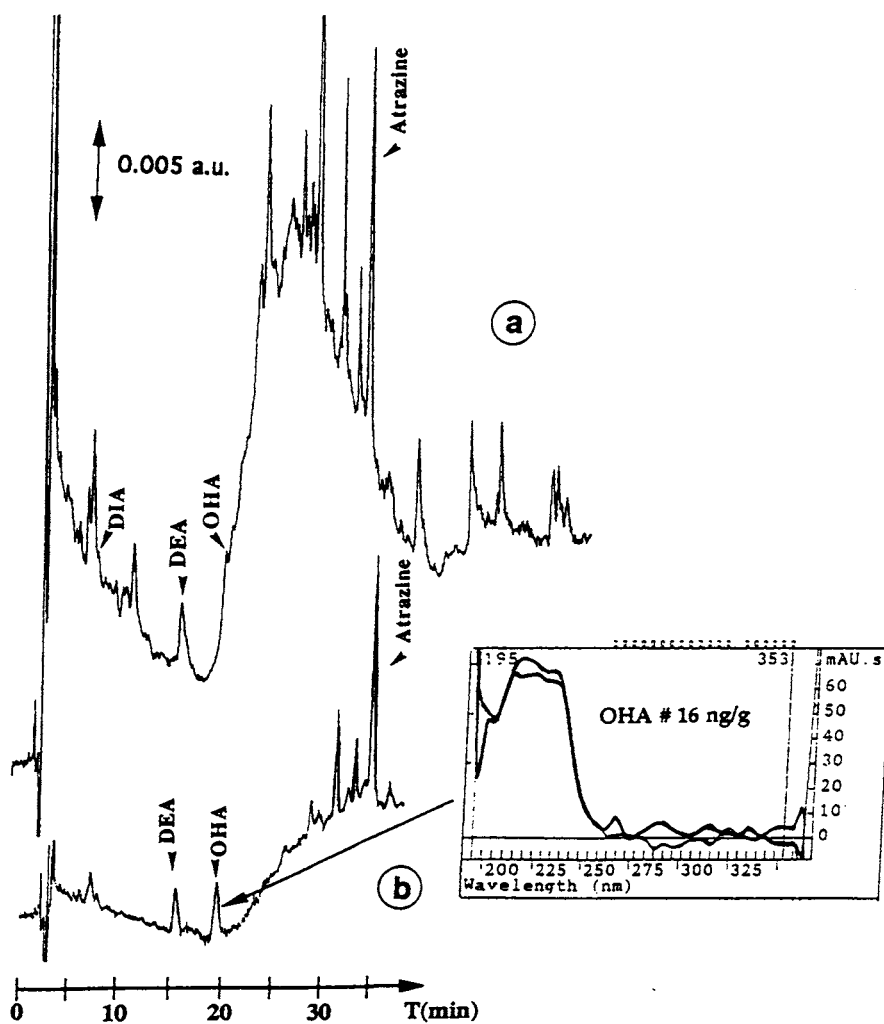
Fig. 1.17. Analysis of the residue extract of a non-spiked soil percolated onto (a) a non-selective sorbent (apolar copolymer PLRP-S) and (b) an anti-atrazine immunoextraction sorbent. The insert represent the match of the UV spectra for hydroxyatrazine. Reversed-phase LC with water-acetonitrile gradient and UV DAD at 220 nm. De-ethylatrazine (DEA) and atrazine identified with respective concentrations of 7 and 23 ng/g (dry soil). From [199].

using a precolumn prepacked with a non-selective polymeric sorbent (PLRP-S). This chromatogram contains many peaks and a huge hump, but it was possible to identify atrazine and some metabolites. De-ethylatrazine (DEA) can be identified but hydroxyatrazine (OHA) is only slightly visible, thereby rendering impossible any quantification. Fig. 1.17b shows the analysis of the same diluted extract, but using precolumn packed with an anti-atrazine immunosorbent instead of PLRP-S. One can see the advantages of the high selectivity of the pretreatment by the easy identification and quantification of hydroxyatrazine which has a characteristic UV spectrum, at concentration as low as 16 ng/g (dry soil). The identification is strongly reinforced by the molecular recognition involved in the clean-up [199,216].

The analysis of phenylureas and triazines in several food samples (carrots, celery, corn, grapes, onions, potatoes, and strawberries) was also shown to be highly simplified [217,218]. Methanolic extracts of the plant tissues were simply concentrated and then diluted with water before passage through the IS. Thanks to the high degree of clean-up, this approach was very rapid compared to actual methods and eliminates the requirements of solvents such as hexane, dichloromethane, acetone and others commonly used for adsorption chromatographic clean-up of sample extracts. PAHs could be determined in waste sludges and sediments using LC-UV DAD. The method was validated using certified reference sludges and sediments [219]. The clean-up provided by an antifluorene IS was shown to be better than that obtained using conventional silica clean-up.

Clean-up using size-exclusion (or gel permeation) chromatography is based on separation by molecular size. Fractionation by polarity using Florisil, silica gel, or alumina selects a limited range of the analytes but does not remove high molecular weight materials of similar polarity. In contrast, size-exclusion chromatography (SEC) primarily removes materials of high molecular weight, leaving all the analytes and other compounds of the same weight in the selected fraction. That is particularly important for matrices containing high molecular weight interferences such as triglicerides in food or humic substances in soils. In the current environmental analysis of pesticides, polystyrene columns are the most used SEC sorbents, and are eluted with cyclohexane, ethyl acetate–toluene, cyclohexane–dichloromethane, or cyclohexane–ethyl acetate. This last mixture is often selected because of its compatibility with the ethyl acetate used for extraction of pesticides in various solid and liquid matrices. A comparative study was carried out using various types of SEC columns for the isolation of the pesticides monuron, linuron, monolinuron, isoproturon, propanil, fenitrothion, molinate, alachlor, trifluralin and atrazine from soil samples [220]. Low-resolution SEC polystyrene columns, Bio-Beads SX-3, SX-8 and SX-12, a high-resolution SEC polystyrene column Phenogel, and a silica-based SEC column Zorbax PSM, were compared. The eluent was optimised for the screening of the pesticides and dichloromethane-cyclohexane mixtures gave the best results.

1.5.2 Clean-up included in the SPE sequence

With hydrophobic sorbents, the clean-up step can be included in the SPE sequence. It is performed by flushing the SPE cartridge with a small volume of water modified with an organic solvent so that many matrix components are eluted, but not the analytes of interest. In fact, this flushing can only remove interferences which are more polar that the analytes

of interest, so the method can only be applied for the determination of hydrophobic analytes. It is difficult to apply it for multiresidue analysis. In such cases, if some polar analytes are also to be determined, it is very important to verify that no loss occurs with this flushing solvent.

Another means for removing some polar interferences is to add some organic solvent to the aqueous sample before percolation through the sorbent. Again, this procedure can only be applied for the determination of non-polar compounds. The breakthrough volume will not be estimated from $\log k_w$ values but from $\log k$ values obtained with that concentration of organic solvent.

1.5.3 Coupling of different sorbents

Fractionation can occur by coupling different sorbents when the clean-up cannot be performed after sample application and before analyte elution. Di Corcia and Samperi [144] have shown that selective SPE can be performed by coupling two sorbents. The first cartridge packed with the non-specific graphitised carbon black sorbent, traps the analyte of interest and many other compounds, but only basic analytes are transferred and re-concentrated into a second cartridge packed with a more specific sorbent such as a cation exchanger. They could then determine chlorotriazines at the ppt level. By percolating of 2 liters of drinking-water through a cartridge packed with 250 mg of graphitised carbon black, and then connecting this cartridge to a second one packed with a cation-exchanger and flushing the two columns with a mixture of dichloromethane and methanol, these authors have also determined 14 phenylurea herbicides in drinking water with detection limits at the ng/l level. Phenylureas are eluted while all basic interferences such as chlor-otriazines, and anilines. are trapped by the ion-exchanger.

The removal of humic acid was performed using strong anion exchanger (SAX) disks [221]. The SAX disk was stacked over a C_{18} disks (method so-called double-disk SPE) and it was demonstrated that humic substances were removed effectively by the SAX disk and were not eluted in the organic solvents used for the isolation of phenylurea and triazine herbicides. An application to real water show the large reduction in the humic peak that can be thus obtained, when comparing to the use a single C_{18} disk.

Layers of aminopropylsilica followed by C_{18} silica have been made in order to remove humic acid, the aminopropylsilica acting as a weak ion exchanger.

The possibility exists also to fractionate the same sample using in series two cartridges, the first one packed with C_{18} silica and the second one packed with high cross-linked SDB polymers. Apolar are retained on the C_{18} silica whereas moderately polar and polar ones are on the SDB sorbent. The advantages of such a fractionation has been described using precolumns in series by Nielen et al., when off-line SDB polymers in cartridges were not available [78].

1.6 ON-LINE COUPLING OF SPE TO LC

Advantages of on-line coupling SPE to chromatographic separations are mainly that no risk of loss or contamination exists and the automation potential. The commercialisation of automatic devices and of sensitive diode array detectors has certainly help for developments in the environmental field. The basic principles of SPE that have been described

above are valuable for the prediction of recoveries. The main differences lie in the selection of the sorbent and in the possibility of coupling several sorbents in series.

1.6.1 Characteristics of the on-line coupling

A description has been represented in Fig. 1.2. The LC system is often run in the reversed-phase mode with C_{18} analytical column and an acetonitrile–water or methanol–water gradient because the residual water in the precolumn does not have to be removed before desorption. By adding a second LC switching valve, there is the possibility of both direct injection in the analytical column and preconcentration via the precolumn. Comparison of corresponding chromatograms indicates rapidly the quality of the coupling precolumn-analytical column. Loss in efficiency of the analytical column can occur due to the coupling and is visible by larger peaks obtained in the on-line chromatogram in comparison with that obtained by direct injection into the analytical column. It should not occur if geometry and packings of the precolumn have been correctly selected.

1.6.1.1 Precolumn size and packings

Band-broadening has to be considered with on-line set-up with precolumn. The size of the precolumn is an important parameter easy to understand because the profile of concentrated species transferred from the precolumn to the analytical column should be ideally as narrow as possible at the beginning of the separation. Consequently, the size of the precolumn should be as small as possible and depends on the size of the analytical column. For a classical analytical column of 15–25 × 0.46 cm i.d. the length of the precolumn should be at maximum 15–20 mm and the diameter lower than that of the analytical column [78]. Our own experience indicated to us that taking a very small precolumn was important if the separation is carried out in with an isochratic mobile phase [16]. With a methanol or acetonitrile gradient, the dimension of the precolumn can be slightly increased and the band broadening can be reduced or removed by applying a gradient of mobile phase. This is why the ability to control the quality of the coupling by comparing direct and on-line chromatograms is necessary.

One serious limitation of the SPE-LC system is that they use small precolumns which therefore contain a small amount of sorbent. In contrast to off-line SPE, increasing the breakthrough volumes of analytes by increasing the amount of sorbent is impossible. Calculations of Table 1.2 have been made with a 1 × 0.2 cm precolumn as used in automatic devices. By increasing the size of analytical column to 25 cm, the size of the precolumn could be increased, but values of Table 1.2 can only be increased up to a maximum of a factor 10. If a higher retention is required the only solution is to select another sorbent providing higher retention volumes in water for analytes of interest. This is why in on-line methodology, it is important to know the retention behaviour of analytes with the different sorbents that can be used.

The packings should be LC-grade quality in order not to decrease the efficiency of the analytical column. But since 5–10 μm packings do not allow high sampling rate, the trend now is to use 15–40 μm packings, and no decrease in efficiency was observed provided the precolumn dimensions should be low. Backflush-desorption should provide better peak shape than forward-desorption. However, in practice backflush-desorption may create

clogging of the analytical column when real samples are analysed. Since only a very slight difference is observed, forward-desorption is commonly used and then the precolumn acts also as guard column.

In theory, the analyte retention should be ideally similar to, or lower than that on the analytical column for a perfect coupling. Therefore, the most efficient system is ideally obtained from a precolumn and an analytical column of the same nature [15,16]. In practice the selection of sorbents in the analytical column is more limited than those in the precolumns. When many compounds are to be separated over a wide range of polarity, their separation requires a highly efficient analytical columns with both water-rich and organic-rich mobiles phases. At present, only C_{18} silica columns meet this requirement. Analytical column prepacked with polymeric styrene divinylbenzene or porous graphitic carbon are very efficient in organic-rich mobile phases but less in water-rich. The compatibility of sorbents for precolumns and analytical columns is important and will be discussed with the selection of the sorbent.

One advantage of the small size of precolumn is that they can be packed with any LC-grade sorbent, even the most expensive ones. In many procedures described, they are re-used several times after regeneration.

1.6.1.2 Non-selective sorbents

Alkyl silicas. Table 1.8 reports the breakthrough volumes of a set of polar to moderately pesticides on commercial precolumns packed with the same amount C_{18} and polymeric sorbents. For a moderately polar analyte such as simazine (log k_w of 3 on C_{18} silica) breakthrough occur rapidly for a sample volume of 23 ml due to the small amount of sorbent in the precolumn. Therefore, applications with C_{18} are for non-polar analytes or when the required sample for detection is low. The on-line set-up was validated from interlaboratory studies for the determination of organophosphorus pesticides [222]. Fifteen moderately polar pesticides could be monitored in environmental water at low

TABLE 1.8

BREAKTHROUGH VOLUMES IN ML (A) CALCULATED ON A 1×0.2 cm i.d. PRECOLUMN PACKED WITH C_{18} SILICA AND (B) MEASURED ON A 1×0.2 cm i.d. PRECOLUMN PACKED WITH PLRP-S

Compound	C_{18}	PLRP-S
Oxamyl	4 ± 1	30 ± 5
DIA	2 ± 1	25 ± 10
Metamitrone	3 ± 1	75 ± 10
Chloridazon	2 ± 1	90 ± 20
Carbendazim	4 ± 1	180 ± 20
Aldicarbe	6 ± 1	250 ± 30
Simazine	23 ± 5	>350
Metribuzine	26 ± 6	>350
Carbofuran	32 ± 8	>350
Atrazine	80 ± 2	>350
Isoproturon	80 ± 20	>350
Terbutylazine	130 ± 30	>350

levels using on-line SPE with a C_{18} precolumn followed by LC-MS with atmospheric pressure chemical ionisation and particle beam mass spectrometry [223]. On-column trace enrichment and LC using a single short (20 mm length) column was optimised for the rapid simultaneous identification and quantification of a wide range of organic microcontaminants in environmental water samples [224]. Several C_{18} bonded silica packings materials provided good results in terms of analyte recoveries, peak capacity, linearity of response and precision. With 15-ml samples, detection limits of 0.1–0.4 μg/l and 0.5–1.1 μg/l were achieved for tap and surface water, respectively. The applicability of the system was demonstrated by the screening of real environmental samples and provisional identification of unknown using LC combined with tandem MS. The same column could be re-used more than 40 to 50 times [225,226]. The same system was combined with tandem mass spectrometry for the rapid study of pesticide degradation [227].

Polymeric and carbonaceous sorbents. PLRP-S and PRP-1 prepacked in precolumns have been available for many years and coupled on-line with conventional analytical C_{18} column in many multiresidue applications [15–18] and examples of relevant applications have been recently reported [228–231]. A selective analysis of the herbicide glyphosate and its degradation product aminomethylphosphonic acid in water was described by on-line SPE-LC electrospray ionisation mass spectrometry. Derivatisation of the products were achieved directly in the native aqueous sample before the SPE and the whole method was fully automated, allowing detection in the concentration range of 0.05 to 3 μl in various types of water [232]. Usually, no visible or just a slight band broadening is observed when using an appropriate gradient for the transfer, because the difference in retention of analytes between these two SDB with low surface area and C_{18} is not too large.

The performance of the Propekt system for multiresidue analysis of a mixture pesticides in drinking water is shown in Fig. 1.18. A precolumn prepacked with PLRP-S was used and the sample volume was 150 ml. In the non-spiked sample, although breakthrough has occurred for de-ethylatrazine, there is no problem in identifying de-ethylatrazine at a concentration of 0.09 ± 0.01 μg/l. Detection limits were lower than 0.1 μg/l for all the analytes, so that drinking water monitoring is possible with the on-line system. The matrix effect was studied and the same experimental conditions using the same device of the Fig. 1.18 were applied to a contaminated river water sample without any clean-up. The corresponding chromatogram is represented in Fig. 1.19. The amount of humic is higher, about five times, and the river sample was spiked at the 0.5 μg/l. For many analytes, the detection limits are far below the 0.5 μg/l level, so that monitoring of surface water for early warning is possible. Several analytes could be identified in the non-spiked river water sample at concentrations lower than 0.5 μg/l.

Table 1.4 has pointed out the large increase obtained in analyte retention when using SDB polymers with higher specific areas. Some studies have compared the breakthrough volumes of polar analytes using 10 × 2 mm i.d. cartridges as those used in the Prospekt automatic device. For deisopropylatrazine, these values are 4 ml when precolumns are prepacked with C_{18} silica, 25 ml with PLRP-S and above 300 ml with Hysphere-1, Isolut ENV + and Lichrolut EN [16,233]. Therefore there is a real interest in using these highly cross-linked polymers in on-line devices for very polar analytes. But then the composition of the mobile phase and the gradient used for the separation and desorption are very important. A study dealing with multiresidue analysis including some polar analytes has shown a good coupling using precolumns prepacked with polymeric sorbents having
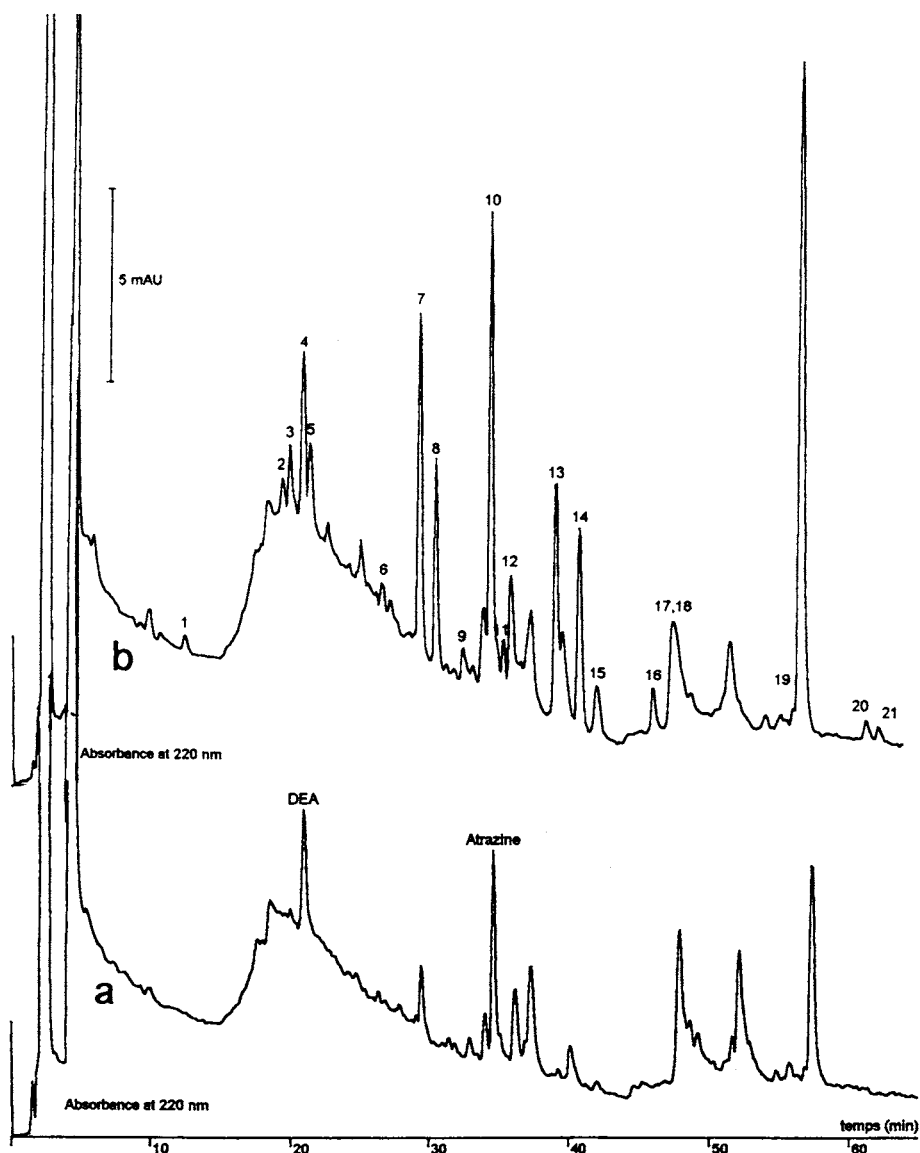
Fig. 1.18. On-line analysis of (a) 150 ml of a drinking water sample and (b) 150 ml of the same sample spiked with 0.1 µg/l of a mixture of 21 pesticides using the Prospekt system. Precolumn: 1×0.2 cm i.d. packed with PLRP-S from Polymer Laboratories; C_{18} analytical column OPDS-80TM, 25×0.46 cm i.d., water acetonitrile gradient: 15% ACN from 0 to 10 min, 50% at 33 min, 50% at 40 min, 48% at 48 min, 70% at 70 min; UV DAD detection, chromatogram at 220 nm. Peak number: (1) Deisopropylatrazine, (2) metamitron, (3) hydroxy-atrazine, (4) deethylatrazine, (5) chloridazon, (6) aldicarb, (7) simazine, (8) deethylterbutylazine, (9) carbofuran, (10) atrazine, (11) isoproturon, (12) diuron, (13) propazine, (14) terbutylazine, (15) linuron, (16) terbuconazole, (17) alachlor, (18) metolachlor, (19) fexoxaprop-*p*-ethyl, (20) pendimethaline, (21) trifluraline. From [16].
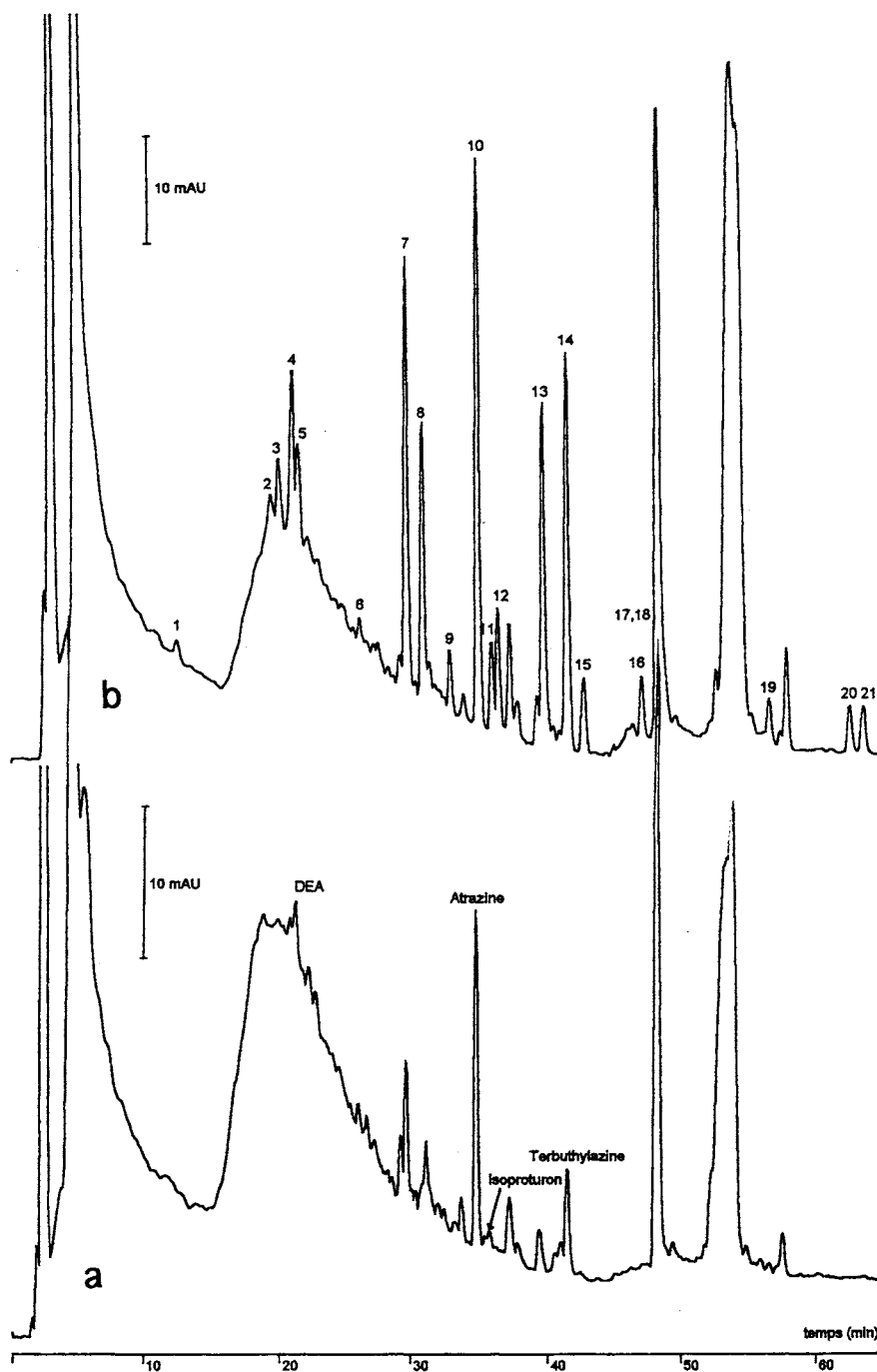
Fig. 1.19. On-line analysis of (a) 150 ml of non-spiked River Marne water and (b) 150 ml of the same sample spiked with 0.5 µg/l of a mixture of 21 pesticides. Experimental conditions and peak identity as in Fig. 1.18. From [16].

specific surface areas of 700 m^2/g (Bondelut PPL) or higher that 1000 m^2/g (Hysphere-1) and an analytical C_{18} silica [135]. Another study dealing with the determination of a mixture containing only very polar analytes has shown a large band broadening when coupling a precolumn prepacked with SDB from J.T. Baker (1060 m^2/g) and a C_{18} silica column, whereas the coupling with PLRP-S was good [234]. In fact that can be easily explained by the shape of the gradient, which is more rapid in the first example than in the second one where the gradient allowing the separation started with 5% acetonitrile, was 15% at 50 min and 30% at 60 min. The coupling depends strongly of the gradient used for the desorption and the separation. In the second example, the mobile phase did not contain enough acetonitrile to allow a good desorption of all the analytes from the SDB precolumn towards the analytical column in a narrow band and then a large band broadening occurred. Backflush desorption only slightly improved the transfer. In the first example, the slope of the gradient was higher because there were few polar analytes mixed with moderately polar analytes as shown by the gradient which was linear from 20% acetonitrile to 40% in 20 min and 100% at 25 min. Therefore, there is no general rule for the apparent compatibility between sorbents and one has to be careful. This mainly depends on the shape of the gradient of mobile phase. A study dealing with the separation of polar carbamates also described a bad transfer using a precolumn packed with Lichrolut EN (1200 m^2/g) [235] whereas another study showed a good transfer for priority phenols [236].

In order to prevent the peak broadening due to the different nature of the analytical column and the precolumn adsorbent, the on-line coupling device can be modified by performing the desorption in the backflush mode, using only the organic solvent of the mobile phase, and then, via a third pump adding water, so that both solvent are mixed before reaching the analytical column [237–239].

In the two examples described above which compared the quality of the on-line coupling using polymer with high specific surface areas, the on-line coupling of precolumns packed with Carbograph [135] and Hypercarb [234] was also studied. The same observations were made: with the water-acetonitrile gradient increasing form 20% acetonitrile to 40% in 20 min, the transfer was correct, whereas with a slow increase in acetonitrile, the transfer is bad. This is illustrated in Fig. 1.20a. When possible, the replacement of the C_{18} analytical column by an Hypercarb analytical column can solve the problem. In Fig. 1.20a the direct injection of 13 polar analytes on a C_{18} analytical column has been represented as well as the chromatogram corresponding to the on-line PGC precolumn/ C_{18} analytical column. The alone analytes aldicarb (peak 10) and metribuzin (peak 12) are transferred with band broadening, whereas the other ones are not desorbed at all. That can be easily explained by the difference in log k_w values as shown in Table 1.9. Fig. 1.20b shows clearly that when both precolumn and column have the same nature, the on-line coupling is excellent and no difference is detected between the chromatogram obtained by direct injection and by on-line preconcentration. [234], but not all the analytes of Fig. 1.20a could be separated using the Hypercarb analytical column.

For the more polar compounds, the on-line system PGC/PGC could solve the accurate determination of the more polar analytes. In a long-term survey of a ground-water source, monitoring using the PLRP-S precolumn/C_{18} analytical column on-line system indicated constant and rather high amounts of atrazine and deethylatrazine with average concentra-
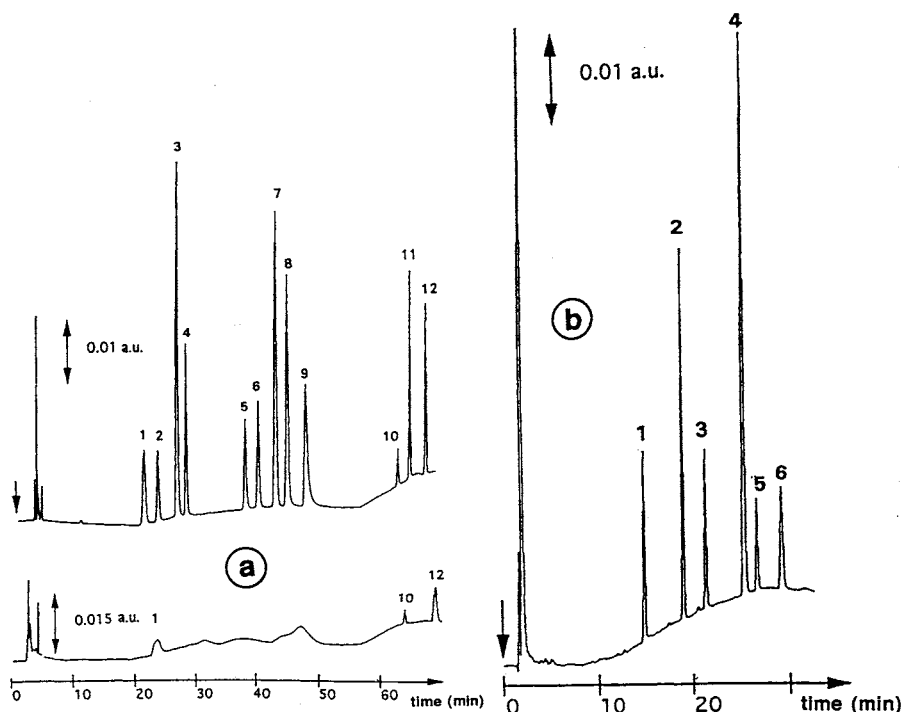
Fig. 1.20. (a) On-line coupling of a PGC precolumn and a C_{18} silica analytical column by comparison with the direct injection of a mixture and (b) On-line coupling of a PGC precolumn with a PGC analytical column. (a) On-line preconcentration of 100 ml of water spiked with 1.5 μg/l of each analyte; C_{18} Supelcosil LC-18-DB (25 × 0.46 cm i.d.), acetonitrile gradient with a 5×10^{-3} M phosphate buffer at pH 7, 5% acetonitrile from 0 to 15 min, 10% at 20 min, 15% from 40 min to 50 min, 30% at 60 min and 65% at 65 min. Flow-rate 1 ml/min; UV detection at 220 nm. Solutes: 1, oxamyl; 2, methomyl; 3, DIA; 4, monocrotophos; 5, fenuron; 6, metamitron; 7, DEA; 8, chloridazon; 9, carbendazim; 10, aldicarb; 11, aminocarb; 12, metribuzin. (b) Water sample of 25 ml spiked with 6 μg/l of each compound; Hypercarb column from Shandon, 10 × 0.46 cm i.d., acetonitrile gradient with a 5×10^{-3} M phosphate buffer at pH 7, 10% acetonitrile from 0 to 5 min, 15% at 10 min, 55% at 25 min.flow-rate: 1 ml/min, UV detection at 220 nm. Analytes: (1) oxamyl, (2) methomyl, (3) monocrotophos, (4) fenuron, (5) de-ethylatrazine, (6) aminocarb.

tions of 0.5 and 0.6 μg/l, respectively [234]. Due to the bad detection obtained with this system for the second metabolite deisopropylatrazine, a PGC precolumn/PGC analytical column was used. Fig. 1.21 shows the advantage of such a system since de-isopropyla-trazine is eluted after deethylatrazine and can be easily delayed to 40 min in the chroma-togram, after the interfering compounds. Breakthrough volume of DIA on PGC is above 100 ml so that detection limits using 100 ml samples are in the low 0.1μg/l in LC-grade water as shown in Fig. 1.21a,b. In the non-spiked ground water (Fig. 1.21c,d), DEA was confirmed at concentration of 0.6 μg/l and the concentration of DIA was 0.05 ± 0.01 μg/l.

On-line determination of hydroxychloroanilines, aminophenols and cyanuric acid was reported using both a PGC precolumn and a PGC analytical column [155]. Hypercarb analytical columns are available up to 10 cm long and even when packed with 5 μm

TABLE 1.9

COMPARISON OF log k_w VALUES ESTIMATED FOR A C_{18} ANALYTICAL COLUMN AND A PGC ANALYTICAL COLUMN (HYPERCARB); log k_w VALUES EXTRAPOLATED FROM THE CURVES log k VERSUS PERCENTAGE OF METHANOL

Compound	log k_w ON C_{18}	log k_w ON PGC
Oxamyl	1.9 ± 0.1	2.3 ± 0.1
Methomyl	1.7 ± 0.1	2.9 ± 0.2
DIA	2.1 ± 0.1	>3.5
Monocrotophos	2.0 ± 0.1	2.9 ± 0.2
Fenuron	2.1 ± 0.1	>3
Metamitron	2.1 ± 0.1	Not eluted
DEA	2.6 ± 0.1	3.2 ± 0.2
Chloridazon	2.0 ± 0.1	>5
Carbendazim	2.2 ± 0.1	Not eluted
Aldicarb	2.3 ± 0.1	2.3 ± 0.1
Aminocarb	2.9 ± 0.2	3.3 ± 0.2
Metribuzin	2.9 ± 0.2	3.0 ± 0.2

particles, they are not as efficient as C_{18} silica columns, especially in water rich mobile phases. If the pesticides of interest are not too numerous, the efficiency of Hypercarb analytical column may be sufficient. The on-line SPE and LC determination of diquat, paraquat and difenzoquat from environmental water was accomplished using the auto-mated on-line device OSP-2 with a precolumn laboratory packed with Carbograph and a Hypercarb analytical column [165]. Sometimes, the on-line transfer in the backflush way was shown to improved the coupling [235].

The on-line coupling of precolumns packed with high cross-linked polymer with such a PGC analytical column have been also described [16,96].

1.6.1.3 Selective sorbents

The on-line coupling SPE-LC is powerful, in general, as shown by the examples above. However, except when the analytes of interest are non-polar, no clean-up of sample can be applied. Therefore, there is a real interest in having highly selective sorbents.

Ion-exchangers. Cation exchangers have been used for the on-line determination of aniline derivatives after the chemical removal of the interfering inorganic cations [166,240]. It was shown that when one is looking for less than 1 μg/l of an organic cation in natural water, problems of overloading the cation exchanger capacity occurred, despite the chemical treatment. For compounds which are ionisable in the pH range 2–10, direct percolation of samples through ion-exchanger can be avoided by using a two trap system which was first described by Nielen et al. [241]. It is based on the fact that solutes are retained on an apolar copolymer such as the PRP-1 sorbent when in their neutral form but not in their ionic form. This was applied to chlorotriazines and their hydroxylated deri-vatives having, respectively ionisation constants around 2 and around 5 [242,243]. The water sample adjusted to pH 7 was percolated through the single PRP-1 precolumn. Then, this precolumn was coupled to a second one packed with the cation-exchanger precolumns
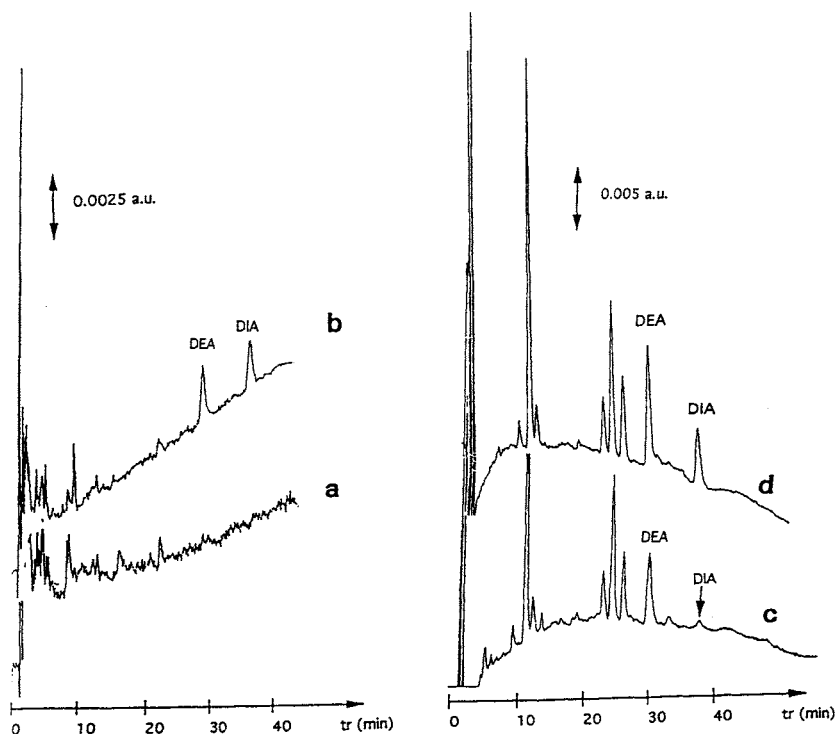
Fig. 1.21. On-line preconcentration of different aqueous matrices using the on-line coupling PGC precolumn/PGC analytical column. (a) 100 ml of non-spiked LC-grade water and (b) spiked with 0.2μg/l of DEA and DIA; (c) 100 ml of non-spiked ground water and (d) spiked with 0.5 μg/l of DEA and DIA. Hypercarb column from Shandon, 10×0.46 cm i.d., acetonitrile gradient with a 5×10^{-3} M phosphate buffer at pH 7, 15% to 35% acetonitrile from 0 to 40 min; flow-rate: 1 ml/min, UV detection at 220 nm. From [16].

and a small volume of well-deionised water containing 25% acetonitrile, adjusted to pH 1, allowed the triazine herbicides to be desorbed from the PRP-1 precolumn, transferred and re-concentrated on the cation-exchanger precolumn. This second precolumn was then eluted on-line. Detection limits below the 0.1 μg/l could be easily obtained in surface water samples. Although no breakthrough had occurred on the PRP-1 precolumn, the use of this single PRP-1 precolumn could not provide these low detection limits due to the numerous interferences. The selectivity provided by the cation-exchanger is illustrated in Fig. 1.22 which shows the on-line chromatogram corresponding to a river Seine water sample spiked with 0.5 μg/l of some chloroaniline derivatives after the two-step preconcentration [244].

One advantage of this two-step preconcentration is that the size of the PRP-1 precolumn can be increased, in order to trap more polar analytes [242,245]. The breakthrough volume of deisopropyl-deethyl and hydroxyatrazine are lower than 100 ml on a conventional 10×0.2 mm precolumn. By increasing the size of the PRP-1 precolumn to 5×0.6 cm i.d. it was possible to detect less than 50 ng/l of these three rather polar degradation products in river Seine water samples.
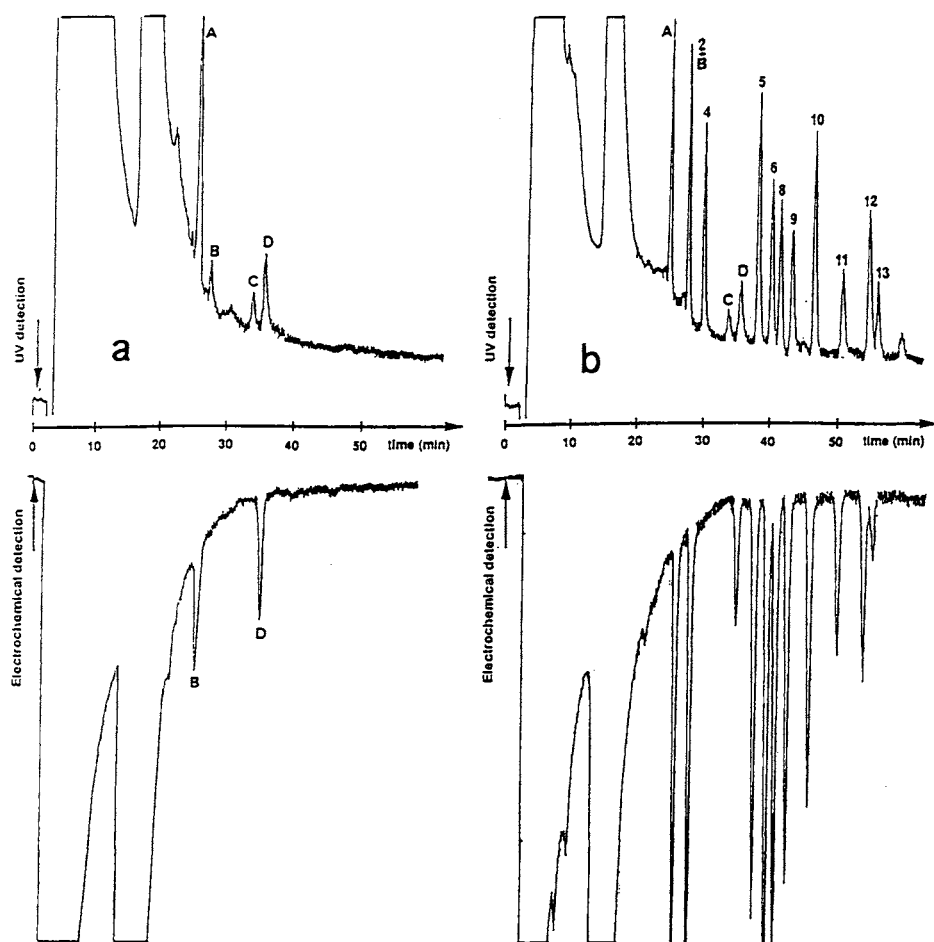
Fig. 1.22. Preconcentration and on-line analysis of a 500-ml sample of River Seine water (a) non-spiked and (b) spiked with 0.5 µg/l of each aniline derivatives. Peak number: (2–4) monochloroanilines, (5–9) chloro-methylanilines, (9–13) dichloroanilines. Preconcentration by percolation of samples through a 15 × 3.2 mm i.d. precolumn packed with PRP-1; then transfer to a second precolumn, 10 × 2 mm i.d. packed with a cation exchanger with 3 ml of de-ionised water adjusted to pH 1 with perchloric acid containing 25% of acetonitrile. On-line desorption of the cation-exchanger content to a C$_{18}$ analytical column (25 × 0.46 cm i.d.) eluted by an acetonitrile-water gradient buffered at pH 5. UV detection at 245 nm, 0.02 µA f.s. Electrochemical detection at 0.95 V (vs Ag/AgCl), 5 µA f.s. From [244].

Anion-exchangers have been also combined to PRP-1 for a two-step preconcentration. Applications to phenol and phenoxyacid herbicides was presented [241,246,247]. Polymer-based ion-exchangers are preferred to silica-based sorbents because of their larger pH range and owing to their higher capacities.

Immunosorbents. One main interest for integrating immunoextraction sorbents in on-line technology is the selectivity of the extraction, because extraction and clean-up are achieved in the same step. So, chromatograms present a clear base-line, allowing quanti-

fication at low level and better identification of the analytes by classical UV diode array detectors. Therefore, the sample volume can be reduced. The on-line analysis of pheny-lureas allowed quantification at the 0.1 μg/l level from a sample volume of 50 ml of surface water (River Seine in Paris) and using a simple UV diode array detector [192]. No interferences from humic acids and other analytes are observed. I. Ferrer et al. combined the selectivity of the IS with the high sensitivity achieved by LC/atmospheric pressure chemical ionisation MS and demonstrated that detection and confirmation could be obtained at the low ng/l from preconcentration volumes as low as 20 ml [248,249]. Other example of the practicality of on-line immunoaffinity extraction techniques for sample clean-up and trace enrichment as a sample preparation for LC/MS/MS with appli-cation has been described in a recent review [250].

ISs can easily be regenerated using a PBS solution, even after being submitted to a high proportion of acetonitrile or methanol. It was verified that after 50 runs the loss in capacity was less than 10% [190,191]. The ISs can be submitted to a high proportion of organic solvent, which destroy the antigen-antibody interaction, probably by deformation of conformation of the protein, but in a reversible way, because antibodies have been stabi-lised by the covalent binding to the silica. However, when possible, it is recommended to switch the valve from the inject position to the load position when the mobile phase reaches 50% to 70% of organic solvent, in order to better preserve the lifetime of the IS.

The on-line coupling IS/LC is particularly appropriate for the trace analysis of mixture containing some volatile analytes because no evaporation of samples occurs. The 16 priority PAHs list of the USEPA contains some 2–3 ring PAHs which are both volatile and hydrophobic. Owing to the low regulatory levels (20 ppt for some of them in surface water which is used for drinking water production) and to the low water-solubility of some 4–6 ring PAHs (as example 2 μg/l for chrysene and 0.3 μg/l for benzo(ghi)perylene), detection of PAHs is required at very low levels. Quantification at the 0.02 μg/l in real matrices requires detection limits in the ng/l range. Due to their hydrophobicity, the addition of an organic solvent (10% acetonitrile) or a surfactant (Brij 35 at a concentration 3×10^{-4} M) in the samples before percolation was necessary in order to avoid the adsorp-tion of the PAHs on container walls or connection tubes [195,196]. Fig. 1.23 shows the chromatograms obtained with an anti-PAH immunoextraction sorbent when spiking surface water with 20 ng/l (20 ppt) of each PAH using LC with on-line fluorescence and UV-diode array detection (UV DAD). Although one fluorescence detector having the capability provided by UV DAD to confirm identity by matching unknown peaks with those in libraries was recently commercially available, in general LC/fluorescence detection suffers from the fact that compounds are only identified by their retention times and their fluorescence properties. Therefore, there is an advantage in having UV DAD in series and a clean baseline for additional identification by the UV spectra which are typical for PAHs. On Fig. 1.23a, one can see the low detection levels which are obtained by fluorescence detection and it was shown that quantification at the required level is possible using a sample volume of only 20 ml [195]. Fig. 1.23b shows the possibility for confirma-tion of identity at this level from the UV spectrum given by the diode array detector for two PAHs. Although some analytes could be confirmed from 20 ml samples, 80 ml are required for identification of all the analytes. Linear calibration curves have been obtained in the range of 0.01 to 0.5 μg/l with excellent correlation coefficients. As the calibration is made through the whole on-line system, by spiking the samples and analysing with the
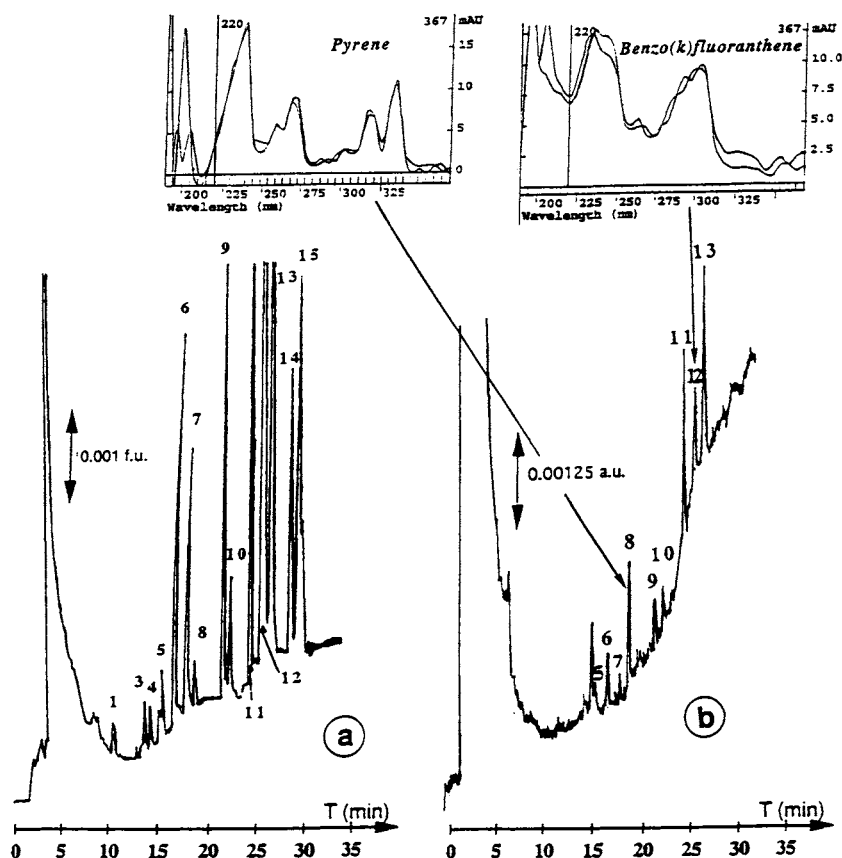
Fig. 1.23. Trace-analysis of PAHs in surface water sample using on-line preconcentration through an anti-fluorene IS (a) fluorescence detection and (b) UV diode array detection in series, with UV spectrum match of pyrene and benzo(k)fluoranthene in the insets. Sample: 80 ml of River Seine water (containing 10% acetonitrile) spiked with 20 ng/l of each PAH.. Reversed-phase LC with water–acetonitrile gradient. Solutes: (1) naphthalene, (3) acenaphthene, (4) fluorene, (5) phenanthrene, (6) anthracene, (7) fluoranthene, (8) pyrene, (9) benzo(a)anthracene, (10) chrysene, (11) benzo(b)fluoranthene, (12) benzo(k)fluoranthene, (13) benzo(b)pyrene, (14) dibenzo(ah)anthracene, (15) benzo(ghi)perylene. From [195].

same experimental conditions as unknown samples, the recoveries do not have to be complete, but have to be reproducible.

Other applications deal with the determination of polar analytes. When sample matrix are more complex such as some industrial effluents are, the detection of polar analytes is hindered by many polar interferences. Due to their carcinogenic nature benzidine and dichlorobenzidines are included in the USEPA Priority pollutants list and in Europe, they are listed among the restricted list of 'very toxic substances for the environment'. These analytes are still produced in large amounts as intermediate compounds in the manufacturing of dyes and pigments. Benzidine is a polar analyte with octanol–water partition coefficient, log K_{ow} of 1.4. An immunosorbent was designed for benzidine and

related analytes using anti-benzidine antibodies [251]. Due to the cross-reactivity of the anti-benzidine antibodies, this IS was shown to be able to extract aminoazobenzene and related azo dyes with good recoveries. The on-line coupling was optimised for the trace-determination of these analytes and Fig. 1.24 shows the chromatogram obtained for the on-line analysis of 2.5 ml of a textile effluent diluted with 47.5 ml of phosphate-buffer saline solution using a precolumn packed with the non-selective SDB polymer PRP-1 (Fig. 1.24a) and the anti-benzidine IS (Fig. 1.24b). The diluted textile effluent has been spiked with 1 µg/l of each three analytes benzidine, 3,3′-dichlorobenzidine and 4-aminoazobenzene. The selectivity provided by the IS is very obvious for such industrial waste which contains many polar interferences co-extracted by the polymer and co-eluted at the beginning of the chromatogram. The quantification and identification of benzidine is easy with the IS and the two other analytes although the wavelength is not appropriate for the 4-aminoazobenzene better detected above 360 nm.
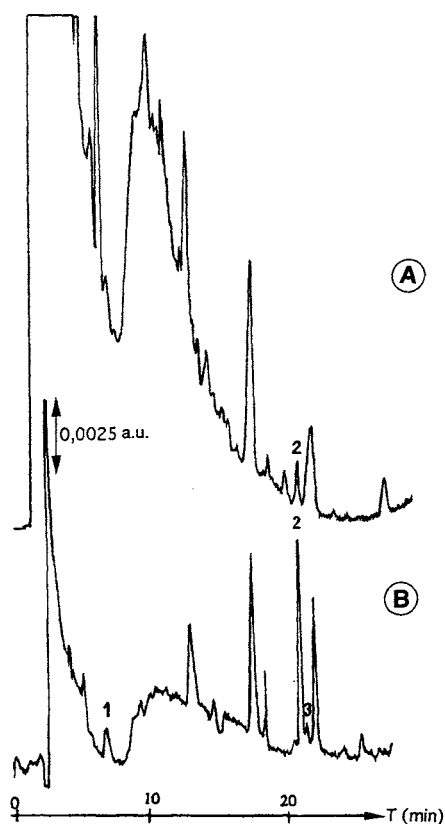
Fig. 1.24. On-line analysis of 2.5 ml of textile effluent diluted with 47.5 ml of PBS using a precolumn packed with (a) the non-selective PRP-1 and (b) the anti-benzidine IS. The 50 ml sample was spiked with 1 µg/l of each analyte: (1) benzidine, (2) 3,3′-dichlorobenzidine, (3) 4-aminoazobenzene. C_{18} analytical column and acetonitrile gradient with 0.005 M phosphate buffer at pH 7, 25% acetonitrile from 0 to 5 min, 47% at 25 min and 80% at 40 min; UV detection at 287 nm. From [251].

1.6.2. On-line sample handling with precolumns in series

The on-line set-up usually is performed with a single precolumn. However there are many examples where more selectivity can be obtained when using two precolumns. Two or three precolumns can be coupled in series during the percolation of the samples with the advantage of pre-fractionation or simplification of a complex mixture during the preconcentration step. One example was already discussed with the use of ion-exchangers for ionisable analytes.

1.6.2.1 Fractionation in polarity groups

A pre-fractionation into polarity groups (non-polar, moderately polar and ionic compounds) was performed using different types of sorbents from series such as C_{18} silica, PRP-1 copolymer and cation-exchanger sorbents [166]. Table 1.10 shows the breakthrough volumes of 29 selected pollutants on various precolumns. Non-polar analytes (nos. 20–29) were trapped on the C_{18} silica; medium polarity compounds (nos. 12–19), not retained by the C_{18} silica were preconcentrated by the PRP-1 precolumn and cationic organic compounds by a cation-exchanger precolumns. The water sample was adjusted to pH 3 before percolation so that aniline derivatives (nos. 1–11) were in their ionic form and were not (or only slightly) retained on both RP18 and PRP-1. In order to avoid the overloading of the cation-exchanger with the high amount of inorganic ions contained in natural waters (such as calcium or magnesium ions, metallic cations, etc.), a chemical pretreatment of samples was performed. Inorganic cationic interferences were removed by precipitation and complexation by oxalate and EDTA. Using an experimental set-up with four switching valves, each precolumn was separately eluted by a continuous buffer-methanol gradient and separated on one C_{18} analytical column with diode-array detection. A chromatogram corresponding to the preconcentration of a 5 ml standard solution containing 200 μg/l of each compound is shown in Fig. 1.25. This fractionation was applied to industrial wastewater analysis. The required detection limits were above 10 μg/l so that the handling of a 10 ml sample was sufficient.

Brouwer et al. [252] have developed a system for the rapid determination of pesticides and degradation products, including aniline, chloroaniline and ionisable analytes. The system contained two precolumns in series which were both packed with the apolar copolymer PLRP-S. The second precolumn was loaded with sodium dodecylsulfate before use, and acted for the trapping of ionic compounds. With 10 ml water samples adjusted to pH 3, the fractionation between neutral and acidic compounds was performed allowing detection in the low μg/l range in tap water.

1.6.2.2 Interference removal

Fractionation can also be performed in order to have a part of the interfering compounds on the first precolumn and the analyte of interest on the second one. The coupling of two precolumns packed with C_{18} silica and with PRP-1, respectively was studied for the preconcentration of phenylureas [253]. On the first C_{18} precolumn, breakthrough values of analytes were estimated to 50 ml. By increasing the sample volume up to 500 ml, about 80–90% were therefore trapped on PRP-1. When handling surface waters containing a

TABLE 1.10

BREAKTHROUGH VOLUMES OF 29 SELECTED POLLUTANTS ON SHORT PRECOLUMNS PACKED
WITH VARIOUS SORBENTS (FROM [166])[a]

No.	Compound	Breakthrough volume (ml)		
		RP-18	PRP-1	Aminex A5
1	p-Aminophenol	0	0	>100
2	p-Phenylenediamine	0	0	>100
3	m-Phenylenediamine	0	0	>100
4	4-Methyl-m-phenylenediamine	0	1	>100
5	o-Phenylenediamine	0	1	>100
6	Aniline	0	2	>100
7	p-Anisidine	0	1	>100
8	p-Nitroaniline	1	10	>100
9	3-Amino-4-ethoxyacetanilide	1	7	>100
10	o-Anisidine	1	6	>100
11	o-Toluidine	1	3	>100
12	Picramic acid	2	>100	–
13	p-Chloroaniline	2	30	–
14	p-Nitrophenol	1	25	–
15	3,5-Dinitro-o-cresol	10	>100	–
16	m-Cresol	1	37	–
17	Nitrobenzene	2	>100	–
18	p-Chlorophenol	2	72	–
19	p-Chloronitrobenzene	3	>100	–
20	Pentachlorophenol	>100	–	–
21	o-Dianidine	10	–	–
22	2-Aminoanthraquinone	>100	–	–
23	3,3'-Dichlorobenzidine	72	–	–
24	3-Amino-9-ethylcarbazole	50	–	–
25	p-Aminoazobenzene	>100	–	–
26	l-Aminoanthraquinone	>100	–	–
27	p-Dichlorobenzene	17	–	–
28	2-Phenylaminonaphthalene	>100	–	–
29	1,2,5-Trichlorobenzene	>100	–	–

[a] LC-water samples containing 250 ppb of test solutes; pH adjusted to 3.0 with perchloric acid; sampling rate 5 ml/min. –, not determined.

large amount of organic material, this fractionation was shown to be useful for quantitative determination lower than 0.5 μg/l. The first C_{18} precolumn acts as an interferent-filter for many apolar compounds, where as the second traps the analyte of interest with a lower background in the base line.

1.6.3 Potential for on-site monitoring

In the framework of the Rhine Basin programme, an automated LC monitoring system (SAMOS-LC for System for Automated Monitoring of Organic compounds in Surface
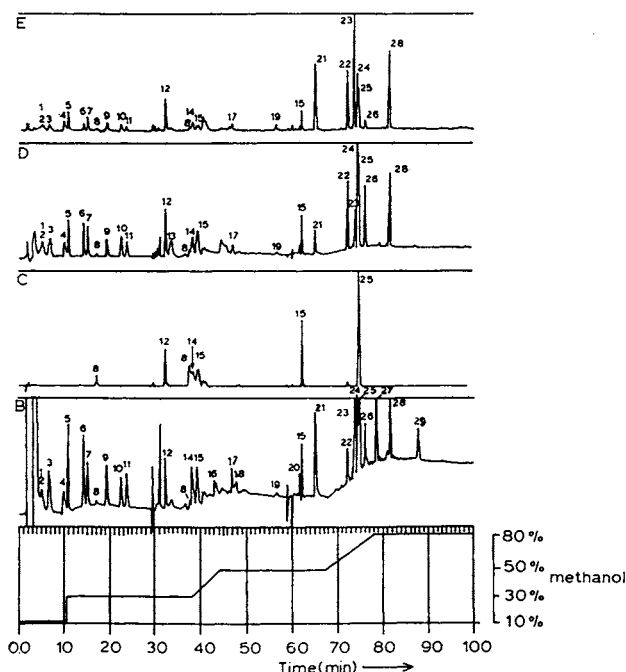
Fig. 1.25. Multisignal plot of a 5 ml standard solution containing 200 ppb of 29 selected pollutants. Gradient elution with 0.1 M potassium acetate (pH 6) and methanol (10–80%) as indicated; preconcentration using three precolumns in series and on-line eluted separately; the first 28 min correspond to the ion-exchanger precolumn, the period 28–58 min to the PRP-1 precolum, and the final period to the C_{18} precolumn; detection at 220 nm (B), 390 nm (C), 244 nm (D) and 295 nm (E), 0.2 μA f.s.; peak numbers correspond to the compounds listed in Table 1.10. From [166].

waters) has been studied extensively [254–256]. The procedure includes the loading of 100–150 ml of surface water onto PLRP-S precolumns of a Prospekt device at 5 ml/min. The on-line analysis was carried out using a C_{18} analytical column with an acetonitrile gradient at pH 3. The data were automatically evaluated, with the production of a report for compounds present at, or above, a certain concentration level between 1 and 3 μg/l [254]. The reproducibility of the retention times with a set of 25–30 pesticides was excellent with a RSD value of 0.2–1.5% ($n = 20$). At an analyte concentration of 1 μg/l, the RSD of peak areas was in the range 1–15%, with a new precolumn in each run. The highest RSD were observed only for analytes eluting between 12 and 25 min and were explained partly by matrix interferences and partly by breakthrough of the more basic compounds on the PLRP-S cartridges [257]. The SAMOS system was made to act as an early warning system for use in the field. The robustness of the system was studied in two laboratories during 5- and 7-month periods. No major problem was encountered for over 1000 analyses, apart from the exchange of a deuterium lamp and clogging of the preconcentration system with non-filtered waters. The system was recently optimised by a new filtration system, the attachment of refrigerated flasks and the use of two precolumns packed with different sorbents [256]. The clogging was reduced permitted an unattended operation for at least 5 days.

The sample handling, transport and storage of samples can be greatly improved because of the small volume of the precolumns. Transport from the sampling site with storage at cool temperature such as −20°C is difficult for water samples in glass bottles. The in-the-field sampling and analyses in laboratory can also be advantageous for monitoring in remote areas. Several studies have examined the stability of analytes sorbed on disposable columns or precolumns depending on the time storage, temperature, type of sorbent, precolumn, presence of water or not during the storage and influence of the pH and sample matrix [71,73,76,256–260]. The stability of nineteen organophosphorous pesticides was investigated on precolumns packed with C_{18} and although acceptable stability was observed for most of the compounds tested, some poor recoveries were observed for unstable organophosphorus such as fenamiphos and fonophos [72]. One can expect a better storage using polymeric sorbents which do not have acidic properties such silanol groups at the C_{18} silica surface. An improvement was observed using polymeric cartridges and fenamiphos was stable for one week at room temperature and fonophos for 1 month at 4°C and at room temperature [233]. Polymeric cartridges were also used for stabilisation of polar phenolic compounds allowing their storage for 2 months at −20°C [261]. The stability of representatives of different groups of polar pesticides sorbed from water samples onto a polymeric sorbent was confirmed during a 7-week period at room temperature or at −4°C [262]. The differences between the stability when stored on wet or dry sorbent were negligible. The pH value of the water samples and a significant influence on stability of only those of compounds that had acido-basic properties.

1.6.4 Quantitative analyses and validation

In off-line SPE-LC, the recoveries should be known since quantitative analysis are made through external calibration curves obtained by direct injections, using the same experimental set-up. This is why it is recommended to have recoveries above 70%. With on-line systems, it is not advisable to carry out quantitative analysis by comparison with direct injections. First, the volume of many injection loops is specified to an average accuracy of 20% and calibration of a loop is a rather delicate and time-consuming operation. This does not have to be considered with off-line procedures because the same loop is used for both analysis of unknown extracts and construction of calibration curves. Secondly, slight but imperceptible band-broadening may occur.

For the above reasons, any quantitation method (calibration curves, standard addition, etc.) should be performed using the whole procedure, i.e. with the same experimental conditions (same types of precolumns, sample volume, analytical column, and on-line gradient elution) as selected for the analysis of unknown water samples. Therefore, it is not necessary to know the recovery of the extraction process for each analyte. When possible, it is better to handle a sample-volume lower than the lowest breakthrough volume for more reproducible results. However, when multiresidue analyses are carried out, the sample volume is selected in order to detect most of the compounds at the required level. With a sample volume of 150 ml and using the Prospekt cartridges packed with PLRP-S, the recoveries of de-isopropyl- and de-ethyl-atrazine are not 100% because breakthrough has occurred on PLRP-S, but it is possible to detect these compounds with reproducible results [263,264].

One advantage of automation in on-line preconcentration is that more reproducible results can be expected, provided the precolumns are packed with the same amount of sorbent and have the same efficiency from one experiment to another one. The overall reproducibility of the method includes both the reproducibility of the preconcentration and of the LC system. The repeatability of peak-areas and heights obtained by direct-loop injections into the analytical column has been studied, using an acetonitrile gradient for the analytical separation. The relative standard deviation (RSD) was between 3% and 7%, and 3% and 5% for measurements of peak areas and peak heights, respectively [263]. In the same study, the reproducibility between cartridges was measured by preconcentrating 50 ml of LC-grade water spiked with 0.5 µg/l of pesticides, using a Prospekt system with a new precolumn packed with the PLRP-S copolymer in each run. The RSD was around 10% ($n = 5$) for measurements of both peak areas and peak heights. RSD values below 10% have been also confirmed in other studies, thus indicating that the precolumns were packed under reproducible conditions. The flow-rate applied for the preconcentration varied from 1 to 5 ml/min and the same average 10% RSD was observed [254].

Validation of the automated on-line solid-phase extraction has been performed by participating in the Aquacheck interlaboratory comparison study organised by the WRC (Medmenham, U.K.) where more conventional sample preparation methods and gas chromatographic determination were being used. The overall RSD between values obtained by the authors and the average value obtained by 14 or 15 other laboratories varied between 1.6% and 36% for atrazine and organophosphorus pesticides in finished drinking waters at levels ranging from 0.02 to 0.2 µg/l [73,222,265].

1.7 CONCLUSION AND FURTHER DEVELOPMENTS

Research into new techniques for sample preparation is a very active area. This is partly explained by the need for reducing as much as possible the use, disposal, and release into the environment of toxic solvents, together with a reduction of the total analysis cost. The key words for any development in area of sample preparation are 'solvent free method'. This is certainly the near end of extensive liquid–liquid extractions followed by silica gel or Florisil clean-up which are still used in many laboratories.

It is clearly established now that SPE can be a powerful method for sample preparation. A proof of its acceptance is the introduction by commercial companies of several new extraction sorbents in both cartridge or disk formats during the past years. Examples are new reversed-phase silicas for polar analytes, polymers with high specific surface areas and various functionalities, and new carbon-based sorbents. The better understanding of the SPE, its study by LC methods and a better consideration of the various type of interactions involved in the SPE process is more and more accepted by developers and end-users. This has led to optimise mixed-mode sorbents or layer cartridges. The trends are also to simplify the labour of sample preparation, increasing its reliability, and eliminating the clean-up step by using more selective extraction procedures. The development of more selective sorbents will remain an active research area, as it is today for molecularly imprinted polymers or immunoextraction sorbents.

In the environmental field the trends are to perform multiresidue analysis with an

increasing number of analytes. Monitoring the environment and preserving the water quality will be a key issue of the next century. Besides well established on-line laboratory methods allowing extraction, separation, identification and quantification of any analyte, automated, on-site and on-line techniques are the priority. Efficient monitoring tools will be required using field methods such as sensors, biosensors and other fields tests. Most of these methods lack of sensitivity or are strongly perturbed from matrix effects in contaminated samples. These methods will gain in interest by coupling simple SPE methods, removing the matrix effect and providing an enrichment factor of 10. Identifying new environmental problems and looking at the responsible compounds or metabolites is another area where SPE will be used differently. There is still an effort to be made in the extraction of very polar analytes in aqueous samples.

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Chapter 2

Separation, clean-up and recoveries of persistent trace organic contaminants from soils, sediment and biological matrices

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CONTENTS

2.1 INTRODUCTION

The number of organic compounds identified as environmental contaminants grows annually and this places the environmental analytical chemist in the dilemma of permanent *catch-up*. As a result, new methodologies and validation programmes are continually required. To resolve this dilemma an agreed priority for the chemical measurement based

on the persistence, distribution and toxicity of the contaminants is required. The effort in the development of environmental analytical chemistry should remain focused on (i) fitness of purpose and (ii) cost-effective chemical methodology and data evaluation.The term 'contaminant' has been used rather than 'pollutant' since the latter should be reserved for those compounds which have a known biological effect. In this paper we discuss the analysis of compounds which come under both categories without distinction.

Considerable resources have previously been put into streamlining and automating the final determination by gas chromatography (GC) and GC–mass spectrometry (MS) coupled with computer-controlled integration, quantitation and data reduction. Until recently many methods used to prepare samples have been labour intensive, macro scale multi-residue schemes containing independent steps of preparation, extraction, clean-up and group separation prior to the final determination. These schemes have attempted to reduce the overall effort of analysis by including as many determinedness as possible in batch analysis to reduce the number of sequential analyses on the same sample. This approach has added value when the amount of sample is limited. However, the recent developments in sample preparation during the 1990s have focused on the optimisation of the analysis of smaller, specific groups of contaminants. The extraction and clean-up stages have been miniaturised and containerised in an attempt to reduce sample preparation time and resources.

These developments have focused on:

- Automation with microwave assisted extraction (MAE)
- Accelerated solvent extraction (ASE)
- Supercritical fluid extraction (SFE) with modifiers
- SFE solid phase *traps* for on-line clean-up
- Coupling of linked liquid chromatography LC–LC, LC–GC and solid phase extraction (SPE)–LC
- Reduction in the use of non-chlorinated and toxic solvents[1]
- Alternative approaches to co-factor analysis of lipids for biological tissue and sieving for sediment
- Methods of preservation and sample handling

These developments in sample preparation during the 1990s have attracted considerable attention by a number of reviewers. Wells [1] provided a review of methods of extraction and clean-up of trace organic contaminants prior to 1993 in the previous edition of this book. Methods of clean-up of pesticides used prior to the mid-1990s have also been reviewed [2]. Norén and Sjövall [3] have reviewed the use of liquid gel partitioning and enrichment in the analysis of organochlorine contaminants in which the lipophilic gel, Lipidex, has been used for extraction and clean-up of a wide range of sample types including milk, fish oil and bile as well as aqueous samples. Sinkkonen [4] reviewed the analysis of chlorinated aromatic thioethers, sulphoxides and sulphones in environmental samples. Polychlorinated dibenzothiophenes and diphenylsulphides are formed

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[1] Whist the authors have reviewed other papers utilising chlorinated solvents and highly toxic solvents such as benzene, this implies no endorsement for their use. It is our view that there are sufficiently suitable solvents/extraction systems now available that these toxic solvents should be phased out of the research and analytical laboratories and this should be reflected in updated methodologies.

during waste combustion and occur in pulp water effluent. The chlorinated sulphoxides and sulphones occur as oxidative metabolites of polyhalogenated hydrocarbons (PHHs) such as polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), poly-chlorinated dibenzo-*p*-dioxins (PCDDs) and Furans (PCDFs). Most of these compounds are co-extracted with the other PHHs and subsequently need to be separated either because they interfere with the identification and quantitation of other PHHs or vice versa. These thio compounds generally occur at a much lower concentration than the parent compounds.

The determination of coal tar and its products have been reviewed by Hale and Aneiro [5]. Many recent developments in sample preparation including SFE, ASE, MAE and solid phase micro-extraction have been applied to coal tar products as contaminants in biota and sediment. De Boer [6] has investigated extraction and clean-up techniques prior to the measurement of PHHs in biota including PCBs, polychlorinated terphenyls (PCTs), chlor-obornanes (CHBs),[2] and tris (4-chlorophenyl) methanol and tris(4-chlorophenyl) methane. Dean [7] focused on extraction methods for environmental analysis including some speci-fic areas in depth. These include the liquid-solid extraction techniques of Soxhlet, includ-ing the Soxtec automated system, shake flask, sonication and steam distillation, SFE, MAE and ASE. The range of compound classes included were PAHs, PCBs, phenols, pesticides, herbicides and phthalate esters. The book gives the background to each technique as well as principles and practice. A selected number of investigations are reviewed in depth and as such it provides a good introduction and teaching aid to recent extraction methods.

2.2 SAMPLE PRESERVATION AND STORAGE

Sampling strategy and methodology, although essential for any meaningful environ-mental analysis, is beyond the scope of this book. Once the sample has been obtained it is essential to maintain the quality of the material in such a way that the final chemical information is representative of the concentrations of determinant in the original sample. This requires a strict protocol for the sample treatment and storage both in the field and in the laboratory.

2.2.1 Biological tissue

Wherever possible the tissue or organ from the species should be obtained immediately following death. This normally means dissection of the individual species in the field. Mobile or containerised field laboratories should be tailor made for these purposes. In many situations the field laboratory should be provided with its own positive pressure filtered air supply to reduce the risk of contamination from the local environment, e.g. on board ship.

Ideally, fresh tissue should be taken directly for analysis, but in most cases the samples need to be preserved and returned to the laboratory. There are a number of guidelines and

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[2] CHBs were referred to as polychlorinated camphenes (PCCs) in this paper. However, there has been a gradual evolution of the nomenclature for these compounds and now the individual Toxaphene congeners are referred to as chlorobornanes (CHBs)

videos relating to the preservation of biological tissue following sampling and the key precautions are given below.

- A clean work area should be maintained and all dissection tools and equipment cleaned between samples.
- Wax-coat surfaces should be avoided. Rolled aluminium foil can be used as a clean surface. A common practice has been to solvent rinse the aluminium foil prior to wrapping or sealing samples for organic trace analysis. However, experience has shown that most proprietary brands of aluminium foil are hot rolled in manufacture and as such provide very clean surfaces. Thus solvent washing will, in many cases, add to the contamination level rather than reduce it.
- Sample should be wrapped in aluminium foil and frozen in liquid nitrogen or individually blast frozen. Tissues should not be collectively frozen in bulk. The time taken to freeze bulk tissues which are wrapped together, often in polythene bags, takes too long and will lead to the deterioration of the samples, especially soft tissues like liver.
- Individually frozen samples should be grouped and stored at $-20°C$.
- The mass of tissue taken for each sample should be limited to the amount required for the analysis. Replicate samples should be stored separately. This practice not only allows small masses to be frozen quickly, but also permits individual samples to be removed from the cold store without the need to thaw more material than necessary. Samples should only be frozen once. Re-freezing significantly affects many biological tissues through cell rupture, loss of fluid and possible alteration of the contaminants.

2.2.2 Preservation of biological tissues

Despite the fact that most laboratories store their biological tissues after sampling, there have been very few detailed studies on the effects of storage on the determinedness, but more importantly upon the tissue matrix itself. Storage under cool conditions in a refrigerator at ca. 4°C is only possible for up to 1–3 days after which there is oxidation of lipids and degradation of the alkylperoxides [8]. The rate at which biological samples are frozen is also important. Slow freezing allows the formation of ice within the cells leading to the denaturing of proteins and a subsequent loss of moisture. A decrease in the lipid content of fish stored at $-30°C$ for ca. 1.5% per year has been reported [9]. Recently de Boer and Smedes [10] have studied the effects of storage conditions of fish at $-5°C$, $-25°C$ and $-70°C$ on CBs, DDT compounds and mercury as well as the lipid content, dry weight, peroxide number and rancidity within the framework of the QUASIMEME[3] project (http://www.quasimeme.marlab.ac.uk). Following this detailed study they conclude that, for the determination of CBs and DDE, storage at $-25°C$ was sufficient for a period of 2 years, but for longer storage periods the temperature should be lowered to $-70°C$ to reduce the effects on the changes to the matrix composition.

Soft biological tissue, e.g. liver, mussels, are normally macerated in a blender or ultra-turrax. Such high speed cutting devices can heat the tissue locally. The container and the material should be kept cold at ca. 0–4°C by immersing in an ice bath. The sample should

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[3] QUASIMEME: Quality Assurance of Information in Marine Environmental Monitoring in Europe. The EU project (1992–1996) now operates as an independent Quality Assurance programme.

be cooled between periods of homogenisation (20–30 s) to prevent excess heating. More sinuous tissue such as muscle may require mixing prior to Ultraturraxing. Some workers add a small volume of water to aid mixing. This approach maybe satisfactory if the tissue is subsequently extracted by blending with the Soxhlet. However, for most extraction methods it is necessary to bind the moisture in the tissue, usually with anhydrous sodium sulphate.

2.2.3 Sediments

Sediments or soils from grab or core samples should be stored either in clean glass jars or in the core tube if the sample is required to be intact. The glass jars are normally sealed with aluminium foil to prevent the inner disc of the container from contaminating the sample. All samples should be wet sieved through 2000 μm mesh and frozen as soon as possible after sampling. The sieving removes grit, small stones, macro-benthos and detritus. This aids preservation by removing the large part of biological material in the sample. Most trace organic contaminants are associated with the fine fraction of the soil or sediment which contains the soot and the organic carbon. Total organic carbon (TOC) is regularly measured in sediments and used as a co-factor with which to normalise the contaminants [11–13]. An alternative approach to using TOC for normalisation which is gaining widespread application is to fractionate the sample by sieving either through a 63 μm or 20 μm mesh sieve.

The advantages of wet sieving are:

- The trace contaminants are concentrated along with the fine fraction and the soot and organic carbon
- A reduction in the heterogeneity of the sample and the variance in the analysis by increasing the concentration of the sample
- This concentration step also reduces the amount of the sample required for the analysis and so making the sample more amenable to extraction methods MAE, ASE and SFE which have a limited container volume.

2.2.4 Drying sediments and soils

Sediment and soil sample are frequently dried following sampling and sieving. This is done primarily to stabilise the material and allow it to be kept in a cool dark store with the minimum deterioration of the matrix and the determinedness. Where the compounds are volatile, e.g. naphthalenes, benzene, toluene, xylenes, chlorobenzenes, it is usually desirable to determine the contaminants directly by extracting the wet sediment. This should be done immediately or by preserving the sample at $-25°C$ or by gamma radiation to sterilise the sediment.

There are three main methods that have been used to dry sediments and soils. These are air or oven drying, chemical desiccation and freeze drying.

Air drying in an oven at ca. 40°C or at ambient temperatures may result in the loss of volatile compounds and increases the likelihood of contamination from the laboratory atmosphere. Most sediments and soils have a high percentage of moisture and it may take several days to produce the dried product. To speed up the process the materials are often spread over a wide area to maximise the evaporation of the water. These exposed surfaces

are ideal for adsorption of contaminants in the laboratory atmosphere [14]. Any problems of contamination can be monitored by using a C_{18} disc which can be left with the drying material to mimic the adsorption onto the drying sediments. The discs are subsequently extracted and analysed. Such confirmatory tests will either provide assurance of the method or show that it is unsuitable.

Campangpangan and Suffet [15] found that air and oven drying resulted in losses of the more volatile PAHs. Chiarenzelli et al. [16] also experienced losses of CBs by evaporation from contaminated sediment. The mechanism of the losses were likened to a steam distillation which stopped when the sample was dried. Anhydrous forms of $CaCl_2$, $MgSO_4$, $CaSO_4$ or Na_2SO_4 have been use as drying agents. Campangpangan and Suffet [15] found that $CaCl_2$ provided the most rapid desiccation. However, the addition of a desiccant increases the bulk of the sample, diluting the contaminants and reducing the effective sample mass that may be taken for some methods where there is an upper limit on the amount of material that may be extracted in any one batch, e.g. containerised methods like SFE, ASV, or MAE or even the Soxhlet if the sample is very large.

Freeze drying rather that lyophilisation is now widely practised and is very reliable, if used correctly. Losses from evaporation are greatly reduced if the samples are kept at a temperature below $0°C$, with no losses for any chlorinated contaminants less volatile than hexachlorobenzene (HCB) [17]. Smedes [18] found no losses of CBs more highly chlorinated than CB18 over a 10 day period with an evaporation temperature of $-5°C$. In addition to using a lower temperature it is advisable to cover each sample in its container with a lid that has a ca. 2–3 mm hole. The water vapour, which is removed primarily through heat transfer to the sample causing evaporation, is not impeded, whereas any contamination can only reach the sample through the small hole by diffusion. This process also allows very contaminated materials to be prepared since each sample is self contained and does not contaminate the inside of the freeze dryer or other samples in the chamber. These two precautions have done much to establish the credibility of freeze drying as the preferred method for removing water from sediments, soils and sewage sludge.

Following the drying process the soil or sediment are ground, ball milled and mixed thoroughly to homogenise each fraction of the sample. It is essential that the material is completely homogenised prior to sampling the specific mass for analysis. This may be achieved by extensive ball milling with agate balls for 8–10 h. The sample, if stored for any time prior to analysis, should be re-homogenised by shaking the container with an agate or Teflon ball inside to re-mix the contents which will have settled.

2.3 RECOVERIES

An essential part of the validation of an analytical method is to obtain the performance characteristics in relation to the precision and bias of the method. Apart from calibration errors, a positive bias is usually related to contamination and interference, while a negative bias is often a function of low recovery of the determinant from the matrix or from losses during sample preparation, e.g. co-distillation during solvent removal. The ideal recovery measurement of a determinant is to compare the analytical value with the value of a certified reference material (CRM). With the general increase in awareness of the need for valid analytical methods (VAM) there has been a welcome expansion in the avail-

ability of CRMs for persistent organic environmental contaminants. However, for many compounds these CRMs are either not available or the matrix type or concentration range is not directly comparable with the laboratory samples. Where a CRM is not available or it is not an appropriate matrix or concentration range, the next best option is to obtain a laboratory reference material (LRM) or a well characterised material which has been used in a reliable interlaboratory comparison or proficiency test. Information on the assigned values of each determinant and the level of uncertainty of the estimate, normally accompany such samples. Evaluation of extraction efficiency and clean-up techniques are best achieved with either a CRMs or an LRM rather than with spiked material where the added determinedness may not be as firmly bound as those in the natural sample.

Performance data for a method are required at a minimum of two concentration levels. These are usually chosen to be in the lower quartile and the upper half of the declared range of the method. A number of replicate measurements (minimum $n = 7$) are obtained over a period of time to provide an estimate of the long term precision of the method. These data can also be used to establish the control charts of a new method provided that the materials used to generate the performance data will be subsequently available to maintain the control charts.

For the initial development studies, however, spiked materials, if made with care, can provide meaningful information without consuming valuable CRMs in the early stages of method validation i.e. if the method does not provide the correct recovery for the spiked sample then it is likely that the recovery will also be unsatisfactory for natural RM. In such cases excess materials from interlaboratory studies can be valuable for these tests. Spiked materials can be used successfully in obtaining meaningful recovery data provided that a number of essential steps are observed.

- The concentration of determinant in the spiked material should be representative of those levels found (expected) in the test samples.
- The matrix type should be selected to be representative of the natural materials. This has the added advantage over CRMs which, although having certified values for the determinant, may not be an entirely satisfactory matrix.
- The *one-off* spiking exercise should be avoided. Little information is obtained about the precision or the bias of a method with a small scale spiking experiment. Sufficient material should be prepared for a number of test evaluations, both within the laboratory and between laboratories in *round-robin* exercises. Although this approach takes time and resources to organise, the data which is subsequently generated is substantially more robust, being part of a collaborative method performance study.
- The wet (moist) sediment should be sieved through 2000 μm and spiked with the determinedness in a water miscible solvent such as acetone or methanol. The material should then be mixed in a cool dark place for a number of days before sampling. If the material is to be sieved further, i.e. <63 μm or even <20 μm then this should be done prior to spiking. The binding and the adsorption of the spiked material into the sediment or soil matrix is both kinetically and thermodynamically controlled. An aged spiked sample more closely resembles the way the contaminants are bound to the natural material. Li et al. [19] undertook spiked experiments to determine the recovery of a wide range of organic contaminants from sand and soil samples. The materials were spiked and only left for 30 min to allow the solvent to evaporate. Good recoveries can

be obtained from such samples, but a matured sample provides a more realistic test material.

- There are now reliable methods for the preparation of wet biological materials which can be homogenised and stabilised for use over a number of years [20]. Although most of these materials have, hitherto, been prepared as natural samples for laboratory performance studies, the same techniques for preparation and testing can be applied to spiked materials.

- Organic contaminants can only be fully bound into biological tissue through feeding or exposure studies. The animal distributes the trace organics throughout the body, partitioning and possibly metabolising the material in the normal way. The dose can also contain a small fraction of a radio labelled tracer of ^{14}C which could be measured directly in a specific tissue after sacrificing the animal. The concentration in the tissue from the scintillation measurement of the radio labelled material can be compared to the amount determined by organic extraction, clean-up and analysis by GC or GC–MS to determine the full efficiency of the method. Such experiments are extremely costly and time consuming and can rarely be justified on the basis of determining extraction efficiency alone.

- Preferably a large (>10 kg) batch of test material should be prepared at any one time. Since these materials will not be used in their entirety for a *single-shot* analysis, there is a requirement to confirm the homogeneity of the material. Once the sample has been sealed in its unit container then 10–15 units should be analysed in duplicate and a simple ANOVA performed on the resulting data to confirm the homogeneity of the material. In such cases it is probably inappropriate to perform these tests on the actual determinedness for which the spiked sample has been prepared. Two alternatives are recommended. An additional set of spiked determinedness can be added for which there are validated analytical methods and these can be used as surrogates for the homogeneity test. It is also useful to measure the total organic carbon as a further test for homogeneity. One of the main difficulties in undertaking these trials for trace organic contaminants is that the level of heterogeneity is usually considerably less than the precision of the analysis of the specific determinant. Hence, by utilising the measurement of the total organic carbon (TOC) which has a higher level of precision it can provide a better estimate of the homogeneity.

- Natural and spiked test materials containing each determinant are preferable for recovery and method performance testing to surrogate standards. Internal standards can be used to control some aspects of the analysis such as the variability in instrument performance. However single or multiple surrogates are often not good indicators of recovery of a wide range of compounds that have quite different physico-chemical characteristics. In specific studies ^{13}C-labelled compounds have been successfully used for a number of years. These are ideal and very specific. However, they are expensive and are unlikely to be available for all determinedness.

2.4 EXTRACTION

2.4.1 Liquid–solid extraction

Liquid–solid extraction (LSE) of organic contaminants from soil, sediment or biota

covers the older techniques of the hot Soxhlet or Soxtec or the cold blender, ultrasonication or shaker. These techniques have been reviewed before [1,7] and are summarised in Table 2.1.

Most of the recent developments of the extraction procedure have been directed towards the miniaturisation, containerisation, and solvent reduction. Since the Montreal Protocol there has been a gradual move to replace chlorinated solvents in analytical methods. Although these solvents were initially selected because they were good and versatile solvents, the di- and tri-chloromethanes and chloroethanes in particular, binary mixtures containing approximately equal ratios of an apolar and polar, non-ionic solvent, can be used with demonstrably equal extraction efficiency. The type of binary mixtures that have been used are:

   toluene:methanol (MeOH)
   diethylether:acetone
   petroleum ether:acetone
   n-hexane:acetone
   n-pentane:acetone
   cyclohexane:ethyl acetate

As alternative extraction equipment has become available, the validation trials have been based on a comparison with the efficiency of the Soxhlet to establish the credibility of the newer techniques. The Soxhlet is regarded as the benchmark, comparative method primarily because it has hitherto been widely available and used as the workhorse for the extraction of organic contaminants matrices. It has also been the main method of extraction in many US Environment Protection Agency (EPA), Food and Drug Administration (FDA) and Association of Official Analytical Chemists (AOAC) Standards Methods.

Lopez-Avia et al. [21] made an extensive evaluation of the Soxtec system for extracting a range of trace organic contaminants including organochlorine pesticides (OCPs), PHHs, nitro-aromatics and haloethers from spiked sandy and clay loam. The study compared hexane:acetone (1:1) and DCM:acetone (1:1) and the effects of matrix type, spike level, addition of anhydrous Na_2SO_4, extraction time and immersion/extraction time ratio in a matrix lattice design. Of the factors investigated, the matrix type, spike level and extraction time had significant effect on performance ($P < 0.05$) for 16 of the 29 compounds tested. The anhydrous Na_2SO_4, and immersion/extraction time ratio has little effect, with the exception of one compound. Both solvent systems gave similar results. A further 64 basic, neutral and acidic contaminants were spiked onto clay loam with three standard reference materials (SRMs) certified for poly aromatic hydrocarbons (PAHs). From the 64 compounds spiked, 20 had recoveries >75%, 22 had recoveries from 50 to 74%, 12 had recoveries from 25 to 49% and 10 had recoveries of < 10%. These results for the recovery of the PAHs were in good agreement with the certified values. This method only required ca. 20% of the volume needed for a conventional Soxhlet or ultrasonication, it was faster and less labour intensive.

Folch et al. [22] investigated the Soxhlet extraction of soils treated with sewage sludge with three solvent mixtures; hexane, DCM and hexane:acetone (41:59). Although it was not possible to distinguish between the efficiency of these solvents, the latter was selected since single, apolar solvents are known to have limited extraction efficiency from some soil/sediments and the second choice was a chlorinated solvent. Chalaux et al. [23] deter-

TABLE 2.1

METHODS FOR THE EXTRACTION OF TRACE ORGANIC CONTAMINANTS IN SEDIMENT AND BIOTA MICROWAVE ASSISTED EXTRACTION

Application	Extractant	Conditions	Reference
PAHs in sludge and harbour sediment	Acetone:water (4:1) or *N*-methyl-2-pyrolidimone	100 °C, 1 h or 130 °C, 1 h	Noordkamp et al., 1997 [51]
PAHs, OCPs, PCBs in soils and sediments	Hexane:acetone 1:1 30 ml	115 °C, 10 min, 1000 W	Lopez-Avila and Benedicto, 1996 [138]
PAHs in HS-3, HS-4, HS-5 SRM 1941	Hexane:acetone 1:1 30 ml	500 W	Lopez-Avila et al., 1994 [47]
PAHs, CBs, chlorinated pesticides, phenols and base-neutrals	Hexane:acetone 1:1 30 ml	10 min 115 °C, 1000 W	Li et al., 1996/1997 [19]
PAHs in SRM 1941a	DCM 30 ml	30 W, 10 min	Letellier et al., 1996/1997 [46]
Hydrocarbons and pesticides in marine sediments	Toluene 6–30 ml, H_2O distilled 1 ml	660 W, 6 min	Pastor et al., 1997 [50]
Pesticides in sediments	Iso-octane:acetonitrile (1:1) water to saturate sample + 2 ml solvent	Five consecutive extractions each for 30 s	Onuska and Terry, 1993 [45]
OCPs in water	DCM 30 ml acetone:hexane (1:1), acetone:petroleum ether (1:1)	500 W, 80, 100 or 120°C	Chee et al., 1996 [139]
PCBs and pesticides in soil	Iso-octane 1 ml, hexane:acetone 1:1 9 ml	1000 W, 15 min, 115°C	McMillin et al., 1996/1997 [51]
CBs in soil (ERA-CRM) and sediments (NRCC HS-1 and HS-2)	Hexane:acetone 1:1 30 ml	115°C, 10 min, 1000 W	Lopez-Avila et al., 1995 [140]
Nonylphenol in water and sediment	DCM 30 ml acetone:petroleum ether (30 ml)	100°C, 50 W; 120°C, 50 W	Chee et al., 1996 [55]

mined nonyl phenols, the metabolites of the nonyl phenyl ethoxylates, in sewage sludge, river and coastal sediment by extracting the matrices in a Soxhlet with DCM:MeOH (2:1) followed by pentafluorobenzylation prior to determination by ECD and MS. Reiser et al. [24] extracted the linear alkybenzenesulphonates and the tetrapropylenebenzenesulphonates from freeze dried sediment in a Soxhlet using MeOH for 15 h.

Morel [25] evaluated ultrasonication, mechanical shaking and Soxhlet with three solvents, hexane, 1,2,2 trichloro-trifluoroethane (Freon 113) and DCM for the extraction of hydrocarbons (n-C_{15} to n-C_{34}) from marine sediments and oyster tissue. Hexane and the Freon 113 were found to have very low extraction efficiency from the marine sediments, while the Soxhlet and mechanical shaking with DCM on the freeze dried sediments gave the best recoveries. The precision of the method ranged from 2% to18%. The best extraction for the oyster tissue was a mechanical shake with methanol (MeOH):DCM (2:3). In reviewing these studies it is difficult to understand why these conclusion should not have been drawn prior to the work. Previous studies [26] have concluded that the extraction power of a single apolar solvent is insufficient except from very fatty tissue containing triglycerides. Apolar solvents are particularly incompatible with the extraction of trace organics bound to a polar matrix such as clay soils, silty sediments and sewage sludge [27]. A simple binary mixture of solvent as a useful starting point offers considerable advantages and has been well tried for the Soxhlet. Chlorinated solvents should no longer be selected for inclusion into new analytical methodologies on environmental grounds. There are sufficiently suitable alternatives, as indicated above.

Soxhlet has been compared with sonication and the shake flask methods and been shown to be more reproducible and effective for a wide range of applications, particularly the more polar pesticides [7]. In general, sonication and shake flask methods were less reproducible than the Soxhlet.

Tavendale et al. [28] used Soxhlet Dean-Stark extractor to isolate chlorophenolics, resin acids and base neutral resin-sources cyclic hydrocarbons from sediment downstream of bleached kraft mill effluents. The wet, homogenised sediment was spiked with surrogate standards in acetone and extracted for 24 h with hexane. The azeotropically separated water was removed, 2-propanol was added and the extraction continued for a further 48 h. Other methods used column extraction [29,30], blending with an Ultra Turrax and ultrasonic extraction [31,32] have also been used.

Calero et al. [33] blended fish muscle with a mixture of n-hexane:diethyl ether (9:1) with a solution of NaCl and H_3PO_4 to extract OCPs and chlorobornanes and Donald et al. [34] used a column percolation of the homogenised fish tissue or sediment with DCM:hexane(1:1) to extract chlorobornanes. Rice [35] studied the levels of chlorobornane homologues in fish using a modified method of Swackhamer et al. [36]. The method itself uses a straightforward Soxhlet extraction with hexane of tissue homogenised with anhydrous sodium sulphate.

Johnston et al. [37] used an ultrasonic probe with acetone and DCM to extract PAHs from coal-tar contaminated soil and Pevin and Uhler [38] used a tissue mixer with DCM to remove PAHs from shellfish (*Mytilus edulis*). Sodium sulphate was added to the homogenised sample prior to extraction to bind the water. Caustic reflux has been used to extract coal-tar related products from sediment and biota. The wet homogenised sample was mixed with KOH and methanol and refluxed for several hours. The saponified products were subsequently extracted with an apolar solvent. However, most of these methods

predate the development of the enclosed extraction systems and are primarily included here for reference [26,39,40–43].

2.4.2 Microwave assisted extraction (MAE)

Although microwaves have been used since the 1970s for the production of metal acid digests, the earliest reported use of MAE for organic contaminants was not until 1986 [44]. MAE systems operate with both open and closed vessels at a frequency of 2.45 GHz. A summary of MAE and ASE applications are given in Tables 2.2 and 2.3.

The open system resembles a Soxhlet style reflux extractor where the samples are sequentially microwaved with increasing power in a covered glass vessel. The main disadvantages are that there is no temperature control, the maximum power for these systems is currently 300 W and the samples are normally extracted one at a time. Onuska and Terry [45] were one of the first to apply microwave extraction to the extraction of pesticides from sediment using an open system. They used a Kenmore Microwave 85962 with ca. 1 g dried sediment which was saturated with distilled water prior to extraction. The solvents tested were iso-octane, acetonitrile, acetone:hexane, benzene:acetone (2:1 v/v), methanol–acetic acid and methanol–hexane. The sediments were extracted for 30 s using maximum power in a Reacti-vial and then cooled in an ice bath for 2–5 min. The extraction step was repeated up to 5 times. Acetonitrile:iso-octane (1:1) gave the highest recovery and it was confirmed that moistening (ca. 10–15%) the dried sediment was essential. Letellier et al. [46] used an open cell system to extract PAHs from sediment soil and air particles. Three certified reference materials (SRM 1941a, SRM 1649a, CRM 524) and two natural sediments were tested. Extraction with DCM (30 ml) for 10 min in a Maxidigest 350 (30–300 W gave good recoveries of >80% with an RDS of < 10%.

In contrast, the closed system provides for up to 12 simultaneous extractions with up to 1000 W of power. The vessels are constructed of stainless steel with an inner lining of PTFE and each system i.e. one of the 12 vessels is fitted with a fibre optic temperature probe and a failsafe system (CEM Corporation, POB 200, Matthews, NC 28106-0200,, USA). Most MAE applications have been made with the pressurised system because of the safety features, the possibility of parallel extraction and the enhanced efficiency by operation under pressure.

Lopez-Avila et al. [47] evaluated the recoveries of 17 PAHs, 20 OCPs, 14 phenols and 13 base-neutral contaminants from 6 standard reference materials using MAE and conventional Soxhlet-Soxtec and sonication. They used closed vessel MAE with acetone:hexane (1:1) at 80°C, 115°C and 145°C for 5, 10 and 20 min. For comparison, the same materials were extracted at room temperature keeping the solvent in contact with the solid. At room temperature the recoveries of the PAH were ~52%, 70% at 80°C, and 75% at both 115°C and 145°C. There was no significant difference in the extraction efficiency with time. The extraction experiments with the phenols and the OCPs gave every indication that MAE was at least as effective as the Soxhlet, but with substantially less solvent and extraction times. However, some of the compounds, e.g. 2,4-dinitrophenol and 2-methyl-4,6-dinitrophenol appeared to have been degraded by catalytic reactions since the recoveries (9.4% and 187.19%) were very low. Following this Barnabas et al. [48] investigated the effect of different ratios of acetone:hexane. The recovery of the PAHs increased with polarity such that 100% acetone (40 ml) at 120°C for 20 min was selected. Chee et al.

TABLE 2.2

METHODS FOR THE EXTRACTION OF TRACE ORGANIC CONTAMINANTS IN SEDIMENT AND BIOTA ACCELERATED OR PRESSURISED SOLVENT EXTRACTION

Application	Extractant	Conditions	Reference
PAHs in urban dust and marine sediment	DCM:acetone 1:1	140 atm, 100°C, 5 min + 5 min static	Richter et al., 1996 [56]
PCDD/Fs in dust, sediment and fly ash	Soaked in HCl (6 M), washed with 5% glacial acetic acid, then extracted with toluene	140 atm, 100°C, total procedure 25 min	Richter et al., 1997 [57]
PAHs in soils	DCM:acetone (1:1)	70–170 atm, 40–200°C, 2–16 min	Saim et al., 1998 [59]
Alkylphenols and alkyl benzene sulphonates in sediment	Methanol	150 atm, 100°C 10 min static + 5 min dynamic	Kreiβelmeier et al., 1996 [62]
PAHs, CBs, chlorinated pesticides in biota and sediments (NIST 1649a, 1650, 1941a and 2974)	DCM, toluene, hexane, acetone and acetonitrile in different combinations	69–152 atm, 100°C, 5 min equilibration and 5 min static	Schantz et al., 1997 [61]
PAHs, PCDD/Fs and chlorinated pesticides in soils and slurries	Acetone:DCM 1:1; acetone:hexane 1:1; toluene	100 atm, 100°C, 5 min equilibration	Popp et al., 1997 [58]
PAHs in NRCC sediment HS-6	Hexane:acetone 1:1 20 or 30 ml	140 atm, 100°C, 5 min equilibration, 5 min static, flush with gaseous nitrogen	Heemken et al., 1997 [60]

TABLE 2.3

METHODS FOR THE EXTRACTION OF TRACE ORGANIC CONTAMINANTS IN SEDIMENT AND BIOTA SUPERCRITICAL FLUID EXTRACTION

Application	Extractant	Conditions	Reference
PAHs in marine sediment diesel soot and air particulate matter (NIST 1649)	CO_2 (100%)	80 or 200°C, 450 atm, 15 min static, 30 min dynamic, 5 ml DCM collection	Young et al., 1995 [73]
PAHs, CBs, PCDD/Fs; organochlorine pesticides; thiophosphate insecticides in Missouri River sediment	CO_2 (100%)	50°C, 1 h static, variable pressure florisil + graphitic carbon trap	Tilio et al., 1994 [141]
PAHs in marine sediment (NIST 1647b + NRCC HS-3)	CO_2 modified with 1 ml toluene for each extraction	15 min dynamic, preheated to 120°C, 400 atm, 60,100, 140°C, 400 ml min^{-1} CO_2	Meyer and Kleiböhmer, 1993 [142]
PAHs in air particulate matter (NIST 1649) and sediments (NRCC HS-3)	CO_2 100%, CO_2:MeOH 9:1, CO_2 modified with HMDS and TMCS[a]	15 min static, 15 min dynamic, 350 atm, 60°C, 500 ml min^{-1} (at 1 atm), 1–4 ml toluene solvent trap	Hills and Hill, 1993 [143]
PAHs in sediments (NRCC HS-3) and sewage sludge (BCR 392)	CO_2 + 500 μl of 1% solutions of TFA, HCit, IPA, TEA and TBAOH[b]	10 min static, 120 min dynamic, 90°C, 400 atm, 250 ml gas min^{-1}	Friedrich et al., 1995 [79]

Organics in sediment (NRCC HS-6)	CO_2 (100%) or CO_2 + 3 × 20 µl MeOH	15 min dynamic, 70°C, 200 atm, on-line coupled to GC/MS	Fuoco et al., 1997 [82]
Triazines + phenyl urea herbicides in dried salt marsh sediment	CO_2 (100%)	500 atm, 150°C	Robertson and Lester 1994, [144]
Triazines in soils, sediments and plants	90% CO_2 10% (McOH:water 98:2 v/v)	300 bar, 45 min dynamic, 1 ml min^{-1}, 65°C	Papillaud and Haerdi, 1985 [145]; Papillaud et al.,1996 [146]
Pesticides in fruit and vegetables	CO_2 (100%)	20.3 min dynamic, 2 ml min^{-1}, 50°C, 350 bar, various traps (C_{18}, diol, Tenax, poropak Q eluted with acetone, MeCN, Et OAC qr MeOA) or solvent traps	Lehotay and Valverde-Garcia, 1997 [147]
Pesticides in meat	CO_2 (100%)	2 h dynamic, 2 ml min^{-1}, 0.4 g ml^{-1}, 95°C, florisil trap at 35°C	Juhler, 1998 [148]
Atrazines in sediment	CO_2 (96%) MeOH (4%) + 2 ml MeOH in flow cell	20 min static, 20 min dynamic, 43°C, 100 atm, 2 ml MeOH solvent trap	Cassada et al., 1994 [70]

[a] HMDS, hexamethyldisilane; TMCS, trimethylchlorosilane.

[b] TFA, trifluoroacetic acid; HCit, citric acid; TEA, triethylamine; IPA, isopropylamine; TBAOH, tetrabutylammonium hydroxide.

[49] used a mixed level orthogonal experimental design to optimise solvent, temperature, time and solvent volume for the extraction of PAH from CRMs (HS-4 and HS-6) marine sediments (given in bold).

Solvent	Temperature (°C)	Time (min)	Volume (ml)
DCM	**115**	**5**	30
Acetone:hexane 1:1	135	15	45
Acetone:petroleum ether 1:1			
MeOH:Toluene 9:1			

The recoveries were between 75 and 95%.

Unlike most workers Pastor et al. [50] used a domestic microwave (Moulinex Super-crousty 1100 W) to extract hydrocarbons C_{10}–C_{32}, PAHs, OCPs and PCBs from sediments. They reported that there were no radiation leaks or over-pressure problems working at 60% power. Barnabas et al. [48] reported electrical arching with large sample and small solvent volume. The problem was solved when the sediment was kept below the level of the solvent. Good recoveries were obtained by Pastor et al. [50] for 2 g of dried sediment with an extraction time of 6 min with 10 ml of toluene and 1 ml water.

Noordkamp et al. [51] compared MAE of contaminated soils and sediments for PAHs with N-methyl-2-pyrolidinone (NMP), acetone, MeOH, EtOH, MeOH-toluene, hydrolysis with KOH, and tetra methyl ammonium hydroxide combined with ultrasonic treatment, rotary tumbling and Soxhlet. Extraction with NMP by microwave at 130°C for 1 h was significantly the most efficient method for the extraction of PAHs from sludge and harbour sediment. The second most efficient extraction was with acetone:water 4:1 at 100°C for 1 h. Hummert et al. [52] used cyclic MAE with n-hexane to extract OCPs and PCBs from marine mammal blubber and pork fat. In a series of spiking experiment they compared MAE with Soxhlet. After only 7 MAE cycles the yield was comparable to that of a 5 h Soxhlet extraction with recoveries in excess of 90%.

Lopez-Avlila et al. [53] made an additional, more comprehensive, detailed study to compare MAE, Soxhlet sonication and SFE for 95 of the EPA listed compounds in Methods 8250. Freshly spiked soil samples and two standard reference materials were extracted in acetone:hexane (1:1) by MAE and Soxhlet, with DCM:acetone (1:1) by sonication and with CO_2 modified with 10% MeOH by SFE. Of the 94 compounds reported, 51 gave MAE recoveries of >80%, 33 between 50% and 79%, eight between 20% and 49% and two <19%. However, these recoveries also include the losses in the subsequent sample preparation. Picoline, for example, only had a recovery of 34% from the blowdown alone. Soxhlet extraction gave similar results with 50 >80%, 32 between 50% and 79%, eight between 20% and 49% and four <19%. Sonication gave slightly higher recoveries with 63 >80%, 25 between 50% and 79%, four between 20% and 49% and two <19%. SFE recoveries were the lowest with 37 >80%, 37 between 50% and 79%, 12 between 20% and 49% and eight <19%. MAE gave the best precision with RSDs of <10% for 90 of the 94 compounds tested. Soxhlet gave the worst precision with only 52 of the compounds having an RSD of <10%. Of the compounds tested, 15 polar contaminants gave poor recoveries with acetone:hexane at 115°C for 10 min (1000 W). When the solvent was changed to acetonitrile the recoveries improved for 10 of the 15 compounds

to >70%. Although this work would appear to favour MAE, it may well be that not all of the other methods, particularly SFE, were fully optimised to produce the best extraction efficiency for the compound tested. Many other workers (see later) were able to obtain good recoveries for many of these compounds with CO_2 modified with 2–3% MeOH.

McMillin et al. [54] found that MAE compared favourably with sonication and Soxhlet for PCBs and OCPs in soil. Their main investigation was centred around the possible labour saving of this extraction method and they concluded that this was only viable if the subsequent solvent reduction and clean-up was also optimised. Two specific practical drawbacks were also noted. There is only one pressure/temperature control vessel in the batch of 12 in the current commercially available systems. If the sample overheats, the pressure may rise to ca. 200–300 psi, rupturing the safety membrane and requiring a repeat analysis. If, however, the sample remains cold then the inefficient extraction will not be noticed until it is registered by a low recovery of the spike or surrogate. To overcome the potential matrix imbalance a small amount of water was mixed with the matrix to allow extraction with apolar solvents [50]. McMillin et al. also checked the potential solvent loss during micro waving, since quantitative transfer following extraction is difficult without solvent washing. Replicate weighed experiments showed <2% loses with acetone:hexane. Materials in the extraction vessels are not normally mixed during MAE. McMillin et al. confirmed that this was not necessary and concluded that some form of mixing probably occurs during micro waving.

Li et al. [19] used a closed-vessel MAE of PAHs, PCBs, base neutrals, OCPs and substitutes phenols from sand, soil, and air filters spiked with the test contaminants. Between 15 g of solid were extracted with 30 ml acetone:hexane (1:1) for 10 min at 115°C and gave recoveries of >80% with an RSD of ca. 10% or better. The potential problem of degradation of thermally labile compounds was tested by measuring the decomposition products of pp'DDT and endrin. The pp'DDT decomposes primarily to pp'DDE and endrin brakes down into the epoxide and endrin ketone, none of which were detected after high temperature and pressure tests at high and low concentrations. Such conclusions do not indicate that this may never be a problem, but that extraction is possible with minimal degradation.

Chee et al. [55] optimised the sample preparation of 4-nonyl phenol (4-NP) in water and sediment using a two level orthogonal array design to compare MAE and Soxhlet. The 4-NP is used in the production of the nonionic surfactants nonyl phenol ethoxylates (NPE). Biodegradation of the NPEs releases the 4-NP which is toxic to marine biota and has been implicated as an endocrine disrupter.

2.4.3 Accelerated solvent extraction (ASE)

ASE is a relatively recent development to improve the miniaturisation, automation and cost-effectiveness of the extraction of trace organics from solid matrices. The technique uses both increased temperature and pressure not only to speed up the extraction process, but also to utilise the changes in the solvation properties of the solute under these elevated conditions. At higher temperatures and pressures the dielectric constant of many solvents increase with a concomitant decrease in viscosity which improves matrix penetration of the solvent and leads to faster and more effective extraction. The commercial ASE 200 system currently available (Dionex Corp., USA) has 24 extraction vessels of varying

volumes from 11 to 33 ml. The sample cell containing the solid is filled with the selected solvent and pressurised up to 2×10^7 Pa and temperatures of up to 200°C for about 5 min. A summary of MAE and ASE applications are given in Table 2.2.

Richter et al. [56] extracted PAHs from reference urban (SRM 1649) dust and marine sediments using 100°C, 1.38×10^7 Pa for 5 min with a 5 min static period using DCM:acetone (1:1) with good agreement with the certified values obtained mainly by Soxhlet extraction. The same group also extracted the monitoring CBs (CB 28, 52, 101, 118, 138, 153, 180) from sewage sludge and from oyster tissue using ASE and comparing it to Soxhlet extraction with hexane for 6 h. Recoveries range from 110% to 160% for the sewage sludge ca. 200 μg kg^{-1} and 86–90% for the oyster tissue at ca. 100 μg kg^{-1}. Richter et al. [57] compared Soxhlet and ASE for polychlorinated dibenzo-*p*-dioxins (PCDDs) and furans (PCDFs) in sediment, urban dust and fly ash. The fly ash was soaked in HCl (6 M), wash with 5% glacial acetic acid and then extracted in toluene. The soils and the sediment were extracted without any pretreatment. The recoveries were very comparable to those obtained with the Soxhlet, but again with significantly lower solvent consumption and time 5 ml ASE in ca. 25 min and 250 ml for the Soxhlet taking 18 h. However, it must be remembered that the waiting time with the Soxhlet should not be equated with the actual labour time. Popp et al. [58] applied ASE to the determination of PAHs, OCPs, PCDDs and PCDFs in contaminated soils from around the copper smelting industry, fly ash and urban soils and it compared well with the routine Soxhlet method used previously. OCPs were extracted with acetone–hexane (1:1) with static times of 2×5 min, 1×10^7 Pa, 100°C with a cell flush volume of 60% of the extraction cell volume, 11, 22, or 33 ml depending on the level of contamination of the sample. After testing with toluene, acetone–hexane, DCM–acetone, they found that toluene provided the best recoveries for PAHs using 2×10 min extraction time. Toluene was also the best solvent to extract the PCDDs and PCDFs, provided the fly ash was pre-treated with acid prior to ASE. Saim et al. [59] investigated the extraction conditions for PAHs from contaminated soils using $0.7–1.7 \times 10^7$ Pa and 40–200°C for 2–16 min with DCM:acetone (1:1). Some PAHs, naphthalene, chrysene and benzo[*b*]fluoranthene were not completely recovered at an operating temperature of 40°C. The optimum conditions found were 100°C 1.4×10^7 Pa for 5 min with 5 min static equilibration. The effects of other solvents were also studied including acetone, acetone:hexane (1:1), MeOH, DCM, acetonitrile and hexane. Only hexane, on its own, gave poorer recoveries, which was not unexpected.

Heemken et al. [60] compared ASE and SFE with Soxhlet, sonication and methanolic saponification for the extraction of PAHs, aliphatic hydrocarbons (C_{12}–C_{30}), PCBs and OCPs in marine particulate matter. The results from the extraction of the CRM sediment and four samples of suspended particulate matter showed that the recoveries and precision of ASE and SFE compared well with the other methods. The same 0.3 g sediment was used as intake mass for ASE with a Dioxex 200 (Dionex GmbH, Germany) and acetone:hexane (1:1) at 100°C and 140 atm for 5 min with 5 min equilibration. SFE (Suprex SFE50, Suprex GmbH, Germany) used 30 min static and 60 min dynamic at 80°C 400 atm with CO_2 modified with 10% MeOH. Saponification used 10 g KOH/200 mg Cu/100 ml MeOH/water (10:1) and refluxed for 2.5 h. The Soxhlet used 150 ml acetone:hexane (1:1) with 200 mg Cu and refluxed for 24 h. Using SFE the average recoveries of PAHs in the three samples ranged from 97% to 105%, for ASE the recoveries were in the range 97–108% compared to the reference methods. Compared to the certified values of the sediment HS-

6, the average recoveries of SFE and ASE were 87% and 88%, most compounds being within the limits of confidence. For the alkanes the average recoveries were in the range 93–115% and ASE achieved recoveries of 94–107% as compared with other methods. The influence of water in the solid particulate matter (54%) on the ASE and SFE extraction efficiency was such that it was necessary to dry the sample with anhydrous Na_2SO_4 to obtain quantitative results.

ASE [61] has been used to evaluate the extraction of PAHs, PCBs and OCPs from reference materials; urban dust-organics (SRM 1649a), diesel particulate matter (SRM 1650), industrial forklift (SRM 2975), organics in marine sediment (SRM1941a), New York/New Jersey waterway sediment (SRM1944), organics in freeze dried mussel tissue (SRM 2974), and ground whole carp (Carp-1 and Carp-2). The ASE conditions were optimised with 100°C at a pressure of between 0.69 and 1.52×10^7 Pa. The solvents evaluated were DCM, acetonitrile and acetone:hexane (1:1). Each solvent system gave good recoveries of most of the determinedness. DCM gave higher recoveries of the higher molecular weight PAHs from the diesel particulates. Good recoveries were also obtained from the carp slurries provided that the samples were completely dried with anhydrous sodium sulphate prior to ASE. The same requirement was placed on Soxhlet extraction. Kreiβelmeier and Dürbeck [62] determined alkylphenols and alkyl benzene sulphonates in sediment using ASE. The analytes were extracted with MeOH at 100°C and 150 atm with 10 min static and 5 min dynamic extraction. The MeOH and the maximum pressure were necessary for the complete extraction of the anionic surfactants, whereas the extraction of the alkyl phenols were independent of both parameters. However, using the current system it was not possible to obtain a quantitative extraction of the alkylphenol ethoxylates.

2.4.4 Supercritical fluid extraction (SFE)

Since the first applications of SFE were published by Zosel in 1978 [63] this extraction technique has developed into a key method for the separation of the contaminant from both sediment and biological matrices. SFE has a number of advantages over classical solvent extractions in that it is faster, more selective and less toxic particularly compared with solvents like DCM which must be handled in isolation from the analyst, e.g. in a fume hood. Camel et al. [64] has reviewed the SFE technique and its suitability to the analysis of environmental matrices. They describe the principles of the method and the coupling to chromatographic clean-up. Hawthorne et al. [65] have also reviewed the factors controlling quantitative SFE and Janda et al. [66] provide comprehensive background and a wide range of applications including PHHs, purgeable halocarbons, triazines, phenoxy herbicides, fuel and crude oil and polycyclic aromatic hydrocarbons (PAHs). Bøwadt and Hawthorne [67] give an extensive review of SFE techniques used in environmental analysis. A summary review of the applications of SFE prior to 1993 [1] is given in the Table 2.2 and also includes the period since that review. Dean [68] has reviewed the application of SFE to PAHs and evaluated the need for modified super critical fluids to improve extraction efficiency, the methods to prevent the restrictor from blocking, the collection of the SFE eluant and the general operation condition. SFE is radically different from the previous extraction methods described here since the main constituent of the solvent system, CO_2, separates from the extractants upon venting to the atmosphere, leaving the

orphaned solutes which are trapped either on a solid phase such as C_{18} or in an organic solvent such as iso-octane or petroleum ether.

The actual configuration of the SFE system is dependent upon the type of material to be extracted, however some general rules for guidance can be given.

- Soils/sediments with sulphur can be mixed with freshly cleaned copper powder or fine copper turnings[4] to prevent the sulphur from carrying over into the extract. The isolation of high levels of sulphur require an additional ca. 20 min static extraction time and an extension to the dynamic extraction time [69]. The additional static time is needed to provide sufficient time for the sulphur and copper to react.
- Whenever possible the soil or sediment should be sieved prior to extraction in order to concentrate the fine material. This will provide for a more concentrated sample and assist the extraction (see above)
- It is important that the sample is dry. Soils and sediment should be freeze dried (see above) and biological tissue should be ground with anhydrous Na_2SO_4 and left for 2–4 h to completely dry prior to extraction. This requirement is similar for other extraction techniques like Soxhlet.
- In general, a test for the efficiency of the SFE is better evaluated with CRMs rather that with spike material (see above).
- The trap temperature must be controlled. An initial setting of 5°C can be used.
- Control of the extraction time, pressure and temperature are critical parameters.

Cassada et al. [70] used SFE to isolate atrazine, de-ethyl atrazine and de-isopropyl atrazine from sediments spiked with ^{13}C-labelled homologues using CO_2 modified with 4% MeOH at 43°C and 1×10^7 Pa with an off-line collection in MeOH down to ca. 0.1 ng g^{-1}. Langenfeld et al. [71] evaluated the effects of temperature and pressure on the SFE of PAHs and PCBs in three CRMs; a river sediment (PCBs), urban air particulate matter (PAHs) and a highly contaminated soil (PAHs). Initially the samples were extracted with pure CO_2 at 50°C and at 200°C. At the lower temperatures the elevated pressure (3.5–6.5×10^7 Pa) had no effect on the extraction efficiency. Good recoveries relative to the certified values were obtained in 40 min from the highly contaminated soil regardless of the temperature. However, the PCBs from the river sediment and the PAHs from the urban dust were only extracted efficiently if the temperature was raised to 200°C. At 200°C the PCBs were extracted efficiently regardless of pressure, while both high temperature and pressure increased the recoveries of the PAHs. Langenfield et al. [71] concluded that temperature was more important than pressure in achieving high extraction efficiencies.

Hawthorne and Miller [72] compared an 18 h Soxhlet extraction with DCM and SFE at low (50°C) and high (200°C) temperatures for railway soil and diesel soot. The samples were mixed with anhydrous Na_2SO_4. The mean recoveries for the 17 PAHs examined in the railway soil was 50% at 50°C, 81% at 200°C and 90% at 350°C. For the diesel soot the recoveries for 13 of the PAHs was 51% at 50°C, 71% at 200°C and 118% at 350°C. Although higher temperatures favoured the better recoveries for the higher molecular weight PAHs, it was also thought that the 2–3 ring PAHs were

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[4] Copper turnings should be degreased by refluxing in solvent, e.g. acetone:hexane (1:1) and then subsequently washed with dilute nitric acid, distilled water and MeOH and stored under MeOH to keep the clean prior to use.

actually generated at these elevated temperatures. So a 30 min extraction at 200°C was selected as the optimum. Later the same group [73] studied both temperature and organic modifiers, 10% MeOH, diethylamine and toluene, using a marine sediment (SRM 1941) diesel soot and air particulate matter (SRM 1649). The best recoveries were obtained with CO_2 diethylamine at 200°C with a 15 min static and 15 min dynamic extraction time. Hawthorne et al. [74] compared supercritical water at 250°C, CO_2 at 200°C and CO_2 with 19% toluene at 80°C to extract PAHs from urban air. Surprisingly the water was generally as effective as the other solvents under these conditions at extracting the PAHs. However, unlike the CO_2 the water had to be subsequently removed from the extract. Barnabas et al. [48] used an experimental design to evaluate temperature, pressure extraction time and the amount of MeOH required as modifier for PAHs in a natural soil. The most critical parameters were the extraction time and amount of MeOH with the optimum conditions being 60 min and 20% MeOH at 70°C and ca. 2×10^7 Pa. Comparative results were obtained by the same group using MAE with acetone as solvent [75], ASE with acetone [76] and DCM, and Soxhlet with DCM. Ashraf-Khorassani and Taylor [77] made a comparison of the modifier addition to the matrix with the more usual approach of adding the modifier to the fluid during the SFE of PCBs in a Standard Reference Material (SRM). Off-line modifier addition was more effective at improving extraction efficiency and the direct spiking required considerably less modifier. This optimised SFE method recovered 20–30% more PCBs than the approved US EPA liquid-solid extraction. Bøwadt et al. [78] studied the solid phase trapping of PCBs with stainless steel beads, silica gel, ODS, and florisil following SFE from dried sewage sludge. The preferred trap materials were the ODS or the florisil with *n*-heptane and with CO_2 and 5% MeOH with a trap temperature of 65°C to provide a relatively clean extract for analysis. Fredrich et al. [79] developed an almost matrix-independent SFE method for PAHs by using binary modifiers which are added to the extraction cell at the time of the extraction rather than continuously in the CO_2 stream. The modifiers triethylamine, trifluoroacetic acid, citric acid, iso propylamine, and tetrabutyl ammonium hydroxide all individually at 1% in toluene were tested on the extraction of CRM 392, sewage sludge and HS 3, marine sediment. The extractions were reproducible and comparatively complete and, with the correct binary modifier, did not require any matrix pre-treatment, e.g. with HCl. Yang et al. [73] also used modifiers to examine the effect on the SFE of PAHs from marine sediment, diesel soot and air particulates and found that diethylamine at 200°C gave the highest recoveries. Fernández et al. [80] used an experimental design to optimise the SFE of PCBs and PAHs from sediment. Under the optimum conditions the recovery of the total PCBs and PAHs were ca. 15% higher with SFE than with the comparative Soxhlet extraction. Notar and Leskovek [81] used a five level spherical factorial experimental design to examine the optimum conditions for the extraction of PAH ring systems 2–6 from sediment with pressure, temperature and methanol modifier. Depending on the nature of the matrix and the concentration of the determinedness, the SFE system can be connected directly to the detector. Fuoco et al. [82] have successfully connected SEF on-line with the GC–MS for the determination of PAHs in marine sediments. Using either CO_2 alone or modified with toluene or MeOH on the extraction cell, the PAHs were cryofocused in the accumulation cell in the GC and then chromatographed directly.

2.4.5 Comparison of extraction techniques

Most recent studies on the extraction techniques discussed in the previous sections have made a direct comparison with the efficiency of the Soxhlet in order to validate the system used, effectively making it the benchmark method. A comparison of the main methods of extraction of trace organic contaminants from sediments and biological tissue is given in Table 2.4.

In reviewing these studies a number of overall observations can be made.

- Using the Soxhlet as the benchmark method of extraction has enabled an extensive intercomparison to be made. It is clear that most of the alternative methods using MAE, ASE and SFE confirm that given the appropriate polarity of solvent the Soxhlet is as exhaustive as any other method. With the exception of a few reports, the modern methods of extraction offer no greater efficiency.
- The compounds that have been included in the extraction efficiency studies were limited to those determinedness which are certified in the materials available. No comparative studies have, hitherto, been conducted on the wide range of other organic contaminants, e.g. CHBs, brominated analogues.
- The extent of the good agreement between data obtained by a wide range of extraction methods would suggest (i) all of the extractable organics were being removed, and (ii) there may not be any significant advantage, in terms of extraction, with any one method.
- Most users of the MAE, ASE and SFE claim that the volume of solvent is greatly reduced. This only becomes significant if a single or double extraction i.e. 2×10^{-15} ml are used. However, in a number of cases, multiple extractions ($n = 7$) are suggested as necessary, with a fresh change of solvent on each occasion. Where this is the case the volume of solvent is approaching that used by the Soxhlet at ca. 100 ml.
- The final major apparent advantage of MAE, ASE and SFE is that the extractions are faster and therefore labour saving. The actual extraction cycle is short for MAE and ASE ca. 5–10 min. However, for multiple extractions there is little time between cycles for the analyst to undertake other tasks and therefore is required to attend the system while in use. The main advantage of the Soxhlet is that once set up it can be left unattended for the full duration of the extraction ca. 8–24 h. In many cases the extent of the labour saving associated with MAE, ASE and SFE is considerably exaggerated.
- Neither MAE or ASE are currently in a configuration that would readily lead to the automation of sample preparation. SFE can be used as an on line system with an in line trap/clean-up which can then be connected to the chromatographic and detection system [82].

2.5 CLEAN-UP AND GROUP SEPARATION

The clean-up and separation of the contaminants into groups is an essential step in providing a robust method. The need to remove the bulk of lipids, wax esters and sulphur from sample extracts prior to GC analysis is well known. However, many of the clean-up techniques used before 1990 either rely on extreme chemical treatment, e.g. concentrated

TABLE 2.4

COMPARISON OF THE MAIN METHODS OF EXTRACTION OF SEDIMENT AND BIOLOGICAL TISSUE FOR TRACE ORGANIC CONTAMINANTS

Method	Advantages	Disadvantages
Soxhlet	Multiple systems possible with banks of 6 or 12 Soxhlets. Extraction is automatic once the system is set up. Hot extraction to improve recovery. No sophisticated equipment necessary (less operator time and training). Very little matrix dependent. EPA methods exist	Relatively large volumes of solvent used. Loss of some volatile compounds unless efficient condensers are used. Solvent penetration limited if the sample is not completely wetted. Thermally labile compounds may decompose. Solvent and extraction thimble purity must be checked. Thimbles require extraction before use. Extraction times from 4–24 h.
Soxtec	Less solvent consumption compared to Soxhlet. Fast extraction compared to Soxhlet. EPA methods exist	High initial cost, some matrix dependence.
Blender/ultrasonic	Simple, inexpensive to purchase and operate. Applicable to a wide range of biological tissue and sediments. Ambient temperature. EPA methods exist. Parallel extractions possible. Cold extraction giving better recovery for some volatile compounds	Labour intensive. Difficult to automate. May not extract contaminants bound to tissue or sediment. Separation of extract and matrix debris necessary. Filters may clog. Limited size of sample. Replicate extraction cycles make the methods more dependent on matrix and more difficult to validate.
Column percolation	Large sample size can be extracted. Cold extraction can be used in parallel. Low equipment cost	Very large volumes of solvent used. High solvent blank. Labour intensive
Microwave assisted extraction	Fast parallel extractions possible (often 12 samples < 1 h). Uses less solvent than Soxhlet. No sophisticated equipment necessary (less operator time and training)	Medium initial cost. Thermally labile compounds may decompose
Accelerated solvent extraction	Uses less solvent than Soxhlet. Rapid extraction (15 min). EPA methods exist	High initial cost for instrumentation. Depending on matrix, higher or lower recoveries were found compared to Soxhlet. Thermally labile compounds may decompose
Supercritical fluid extraction	Relatively fast, depending on the temperature and pressure. Uses non-toxic solvents. Can be semi-automated. Well controlled, reproducible conditions. Extraction times 30–60 min	SFE gas and modifier purity must be checked. High grade SF essential. Limited size of extraction vessel. Care on recovery from extraction vessel. Losses can occur. Lower reproducibility due to replicate extractions. Blockages through the restrictors. Trend for a low recovery of high molecular weight compounds. Relatively sophisticated equipment (needs more operator time and training). Parallel extractions are possible but not standard

H_2SO_4 or MeOH/KOH or fairly low resolution adsorption or size exclusion chromatography.

Interfering compounds fall into two categories; (i) those that affect the subsequent high resolution chromatography, and (ii) those which co-elute with the determinant of interest, e.g. chlordanes and chlorobornanes.

One approach has been to use more sophisticated tandem detector systems such as MS-MS. Although it is possible to obtain low-level measurements (fg) of specific contaminants in relatively unclean extracts with such instrumentation. It is not a sound approach to analyse uncleaned extracts with most instruments used in routine analysis. Also, facilities such as MS–MS are often not available in a number of routine analytical laboratories. In practice, the clean extract is considerably more stable and less prone to deterioration for a wide range of persistent compounds. Changes with time can also affect both co-extractants and determinedness in the dirty sample. Since these samples are often irreplaceable, it is important to use a method which will enhance the preservation of the extract. Co-extracted materials if not completely removed will, over time, degrade the GC injector with deposits from non-volatile components. Components which are sufficiently volatile enter the high resolution column coating the surfaces, modifying the chromatographic phase either temporally or permanently, e.g. first 2–3 m, and changing the retention indices of the determinedness. Excess co-extracts inside the detector can give rise to false negative peaks, due primarily to ion suppression in the MS ion source or the ECD cell, even though the interfering material itself is not observed as a peak [83] The high resolution GC and MS are expensive capital items and have a relatively high maintenance cost associated with their use. Therefore, a clean extract is essential to maintain trouble free operation and to reduce routine downtime.

2.5.1 Measurement of lipid

Lipids are extracted from biological tissues to varying degrees with most solvent systems. The extent of the lipid extraction is dependent on the type of lipid and the composition of the lipid, e.g. the proportion of phospholipid and the triglycerides, the solvent composition and, to a lesser extent, the method of extraction. SFE was developed with the expectation that the differences in polarity between many of the contaminants and the lipids might be sufficient to obtain separation at the extraction stage if only CO_2 were used. However, this has not been completely possible, particularly with the addition modifiers to aid extraction.

A measure of the lipid composition and content is required for two reasons. Firstly, most persistent organic compounds are associated with the lipid in the tissue which is used as a co-factor when assessing the extent of the contamination. Therefore, an accurate measurement of the lipid content is required. Any variance in the measurement of the lipid becomes as important as the uncertainty of measurement of the contaminant. Secondly, a measure of the lipid content is also used to adjust the sample mass extracted or the aliquot of the extract taken to clean-up on the adsorption columns. All adsorption columns, such as alumina, florisil or silica gel, have a finite capacity for lipid, above which the chromatographic separation breaks down and, if the amount of lipid loaded is increased still further, then the lipid itself will break through into eluate [84].

The measurement of lipid is operationally defined. The initial benchmark method has

been that of Bligh and Dyer [85] used MeOH and chloroform to obtain a measure of total lipid in foodstuffs. Since then this method has been modified by countless users and applied to a wide range of both fatty and lean tissues. Booij and van den Berg [86] compared different solvent mixtures for the extraction of the lipids from marine biota and concluded that the chloroform used by Bligh and Dyer could be adequately replaced by DCM. Le et al. (1996) [87] refined the method using chloroform-methanol by extracting in an Eberbach blending jar. The variables examined were the solvent ratio, solvent to sample ratios and the phase separation time. The precision of the method was $< \pm 0.5\%$. Although this method offers an improved precision and ease of operation, it still utilises chlorinated solvents.

Smedes [88] has developed a new method in which the tissue of known water composition was extracted by blending with an Ultra Turrax for 2 min with isopropanol and cyclohexane. A fixed volume of water was subsequently added and the mixture blended for a further two minutes. The phases were centrifuged and the organic phase removed. A further Ultra Turrax mixing with the isopropanol and cyclohexane was made and the organic extracts were combined and the solvent removed. The lipid extract was determined gravimetrically. The Smedes method was evaluated in a between-laboratory method performance study within the EU Project QUASH[5] using mussel (2.3% lipid, CV = 9%), plaice (1.2 % lipid, CV = $\pm 10\%$) and herring (10.3%, CV = $\pm 7\%$) tissue. The sample types provided a range of lipid levels and lipid type. For each sample, the between laboratory agreement for the Smedes method was better than the laboratory's own total lipid measurement based on the Bligh and Dyer [85].

Three conclusions can be taken from these studies:

- Smedes method is robust and applicable to a wide range of sample types
- Total lipid can be measured without the use of chlorinated solvents
- Reliable data can be obtained for lipid measurements if a common operationally defined method is followed

2.5.2 Removal of lipids

The techniques to remove lipids fall into two categories; destructive and non-destructive. They are well developed and have changed very little in recent years with the exception of automated methods that have generally used different configurations of HPLC to replace the gravity columns [1]. A number of these techniques are applicable to a wide number of persistent contaminants, but have been documented elsewhere specifically with PCBs in mind [89] (Tables 2.5 and 2.6).

2.5.3 Sulphur removal

Sulphur is generally no longer a problem to remove from the matrix extract and can either be done during extraction or subsequently as part of the clean-up of the sample [90].

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[5] QUASH: Quality Assurance of Sampling and Sample Handling, EU Project 1996–2000, was a sequel to the QUASIMEME Project Quality Assurance of Information in Marine Environmental Monitoring in Europe which was an EU project (1992–1996) and now operates and an independent Laboratory Performance Study for marine chemical and biochemical measurements worldwide.

TABLE 2.5

METHOD FOR THE SIZE EXCLUSION CHROMATOGRAPHIC CLEAN-UP OF TRACE ORGANIC CONTAMINANTS IN SEDIMENT AND BIOTA

Application	Column	Eluant	Reference
PAHs, nitro PAHs and derivatives in smoked foods	Biobeads SX 3 340 × 25 mm	Dichloromethane:cyclohexane 1:1	Larsson et al., 1988 [116]
PAHs and chlorinated hydrocarbons in sediment and biota	100 A SEC column	Dichloromethane	Krahn et al., 1989 [149]
Alkyl biphenyls, linear alkyl benzenes PCB replacements in sediment and fish	BioBeads SX 3		Peterman and Delfeno, 1990 [150]
Chlorobiphenyls in animals fat	Bio beads SX 3 600 × 25 mm	Ethyl acetete:cyclohexane 1:1	Tuinstra et al., 1990 [151]
Chlorobiphenyls in reindeer, fish l and seal	Bio beads SX 3 50 × 10 mm	n-Hexane	Haglund et al., 1990 [122]
Chlorinated terphenyls Aroclor 5432 and 5460 in fish lipid	Bio beads SX 3 SX 8 600 × 25 mm	Dichloromethane:cyclohexane 1:1	Hale et al., 1991 [152]
43 Organophosphorus pesticides in plant and animal tissue	Bio beads SX 3 300 × 25 mm	Hexane:ethyl acetate 60:40	Holstege et al., 1991 [153]
Chloro and bromo hydrocarbons in biota	300 × 25 mm	Dichloromethane:hexane 1:1	Jansson et al., 1991 [32]
OCPs and OPs in fish tissue	Bio Beads SX 3 450 × 10 mm	Ethyl acetete:cyclohexane 1:1	Porte et al., 1992 [154]
PAHs and OCPs in oysters	Zeolite ZSM-5 in Pasteur pipette	Pentane	Fisher et al., 1993 [94]
Nitrogen and phosphorous containing pesticides in marine sediments	Bio Beads SX 3 (300 × 10 mm) or Polygel HPGPC (300 × 7.5 mm)	n-Hexane, Cyclohexane, Ethyl acetate, Bis-isopropyl ether	Bester and Hühnerfuss, 1997 [92]
Chlorobornanes in water, sediment and fish	Bio beads SX 3	Dichloromethane:hexane 1:1 and 15:85	Donald et al., 1998 [34]

TABLE 2.6

METHODS FOR THE ADSORPTION AND REACTION COLUMN CLEAN-UP OF TRACE ORGANIC CONTAMINANTS IN SEDIMENT AND BIOTA

Application	Solvent	Column	Reference
Toxaphene in biological tissue	*n*-Hexane, diethyl ether	Sulphuric acid	Jansson and Wideqvist [155]
PAH in crude oil and sediments	*n*-Hexane	SPE florisil	Garrigues and Bellocq [156]
Pyrethroids in fish eggs	Acetonitrile:water, Hexane	SPE C$_8$ Alumina/silica gel	Bolygó and Hadfield [157]
Polychlorinated diphenylethers in human adipose tissue	*n*-Hexane	Sulphuric acid, modified silica	Stanley et al. [158]
Tetra chlorobenzyl toluenes (Ugilec)	*n*-Hexane	Basic alumina, silica gel	Wester and Van der Valk [159]
PAH in sewage sludge and soils	Dichloromethane	SPE florisil	Wild et al. [160]
PAH in sediment	Methanol:dichloromethane 2:3	SPE silica	Morel et al. [117]
Toxaphene in fish tissue	*n*-Hexane, *n*-Hexane:diethyl ether 75:25	Basic alumina, silica gel	Van der Valk and Wester [161]
Organochlorine pesticides in milk	Milk:methanol 1:1	SPE C$_{18}$	Redondo et al. [162]
Organochlorine pesticides in animal feed	Acetone:water 1:1	SPE C$_{18}$	Torreti et al. [163]

Mercury has been used in the past, but should be discouraged. Clean copper powder or fine turnings are very effective either in the extraction vessel, e.g. as with many SFE applications or subsequently in the clean-up columns.

2.5.4 Size exclusion chromatography

Size exclusion chromatography (SEC) or Gel Permeation Chromatography (GPC) has also been developed to remove macromolecules such as humic acids, and lipids from small molecules ca. >400 Da in extracts of sediment, soils and biological tissues. Most of the applications using SEC have utilised the Biobeads SX 3, 350 × 25 mm i.d. 200–400 mesh with either cyclohexane or cyclohexane:ethyl acetate as solvent. Table 2.5 gives an overview of the techniques that have been used.

The relatively large columns have a high lipid capacity. However the resolution of these columns is generally quite low. The dual effect is to provide an incomplete separation of the low molecular weight lipids and the contaminants with little or no separation between any of the contaminants themselves. Rimkus et al. [91] have partially addressed this problem by using the SEC column in series with an automated on line solvent evaporation and fractionation on a normal phase Hypersil 5 μm HPLC. This has been successfully applied to OCPs, PCBs, CHBs, nitromusks and miscellaneous contaminants such as bromocyclene, pentachloroanisole and octachlorostyrene. The first column removed the bulk of the lipid while the second column removed the final traces of the lipid and provided some group separation of the organochlorine contaminants.

The resolution of the SEC column has recently been improved with the availability of the High Performance Polygel GPC 5 μm column (300 × 7.7 mm i.d.) (Polymer Laboratories, Amherst, USA). Bester and Hühnerfuss [92] compared two methods of clean-up of organophosphorus and organonitrogen pesticides using gel permeation chromatography, one being a macro GPC (Biobeads SX-3) and the other based on the High Performance (HP) column. Different solvent systems were tested including n-hexane, cyclohexane, bis-isopropyl ether, ethyl acetate and mixtures of these solvents. The preferred conditions selected were cyclohexane:ethyl acetate (1:1) at a flow rate of 1.5 ml min^{-1}. The HP-GPC based on a Polygel 5 μm particle size separated both elemental sulphur and macromolecular humic compounds from the pesticides in 15 min. Recoveries were reported at 60% with a limit of detection (LOD) of ca. 10 ng kg^{-1}. Rimkus and Rummler [93] have also used the HP-GPC column to clean-up and separate chlorobornane congeners (CHBs) in fish tissue extracts. SEC has been applied to different target compounds. Fisher et al. [94] use a zeolite column ZSM-5 (Conteka, Delfzijl, The Netherlands) to remove oxygenated compounds co-extracted from oysters. The zeolite was cleaned by heating to 500°C for 2 h and then filled into a gravity column and the OCPs and PAHs eluted with n-pentane.

2.5.5 Adsorption columns

Adsorption columns using alumina and silica gel were amongst the first to be used to clean-up extracts from biological tissue and sediment [84,95,96]. Florisil has also been used extensively [50,97,98]. The primary application of alumina and florisil has been to remove the co-extracted, polar materials while silica gel has provided group separation of n-alkanes, PAHs, OCPs and CHBs [47,99–101].

The degree of separation on the silica is controlled by (i) the mass of the adsorbent,

usually between 3–5 g, (ii) the polarity of the solvent and (iii) the activity of the silica. The solvent is usually apolar, e.g. pentane or hexane, at the start of the elution, switching to binary mixtures such as diethyl ether-hexane, methyl tert-butyl ether-pentane to remove the more polar OCPs and CHBs. The activity of the silica gel can be anything from 0% to 10% water, depending upon the application. One of the main difficulties with this method is to maintain a constant activity of the adsorbent, especially with the lower percentages of water ca. 0–3%. Under these circumstances the materials are normally kept in a sealed container or used immediately following preparation. The disadvantage of using these absorbents is that the method is labour intensive in preparation and use. It only provides a low resolution chromatographic separation and requires between and sometimes within batch calibration. However, these materials are relatively inexpensive and are ideal for one-shot clean-up. One of the main reasons for their continued use has been that they are disposable and simple to use. Once used, it is currently not possible to regenerate the material to it's original state either by washing with solvent or by heating.

Most of the studies undertaken have been to optimise the activity, solvent polarity and mass of adsorbent (Table 2.6) There have been few recent innovations using the straight classical adsorption materials

2.5.6 Solid phase extraction (SPE) clean-up

Miniaturisation of the conventional clean-up adsorption columns has been possible with the development of the SPE cartridges. Although more expensive per unit, the cost per column should be off-set by the gain in preparation time, reduction in the amount of solvent and the ability to readily combine columns of different polarity and reactivity. The SPE columns are also a prelude to automation, particularly with LC.

Schenck et al. [102–104] have developed a SPE method for the clean-up of lean (5 g) and fatty fish (2.5 g) tissue, respectively for the determination of OCPs and CBs. The samples were blended with a Polytron at medium speed in acetonitrile. Once blended, the supernatant was decanted, diluted with water and passed through a C_{18} column. The column was dried with anhydrous Na_2SO_4, connected to an SPE Florisil column and both columns eluted with petroleum ether. Breakthrough of lipids due to column overload was overcome by passing the eluate through SPE silica. The method for 27 pesticides and CBs compares favourably with the current AOAC [105] and PAM methodology [106]. Florisil SPE columns have been evaluated to obtain the most favourable eluant for a range of 24 pesticides in a range of materials from low fat crab meat to whole milk extracts. Schenck et al. [103] found that 2% diethyl ether in petroleum ether was ideal for removing the pesticides whilst retaining a high percentage of the co-extracted materials. Muccio et al. [107] used a single step partition between *n*-hexane and acetonitrile on a two cartridge system composed of Extrelut-3 with a C_{18} cartridge connected downstream to separate OCPs from oils and fats. The extract was cleaned-up with a florisil mini-column prior to GC. The fat carry-over was of the order of 4–40 mg per 1 g fat. HPLC has been used for the clean-up of sample extracts prior to the determination of PAHs. Fernandez and Bayonona [108] fractionated PAHs, ketones and *N*-heterocyclic compounds in sediment extracts with a semi-preparative silica. Although the HPLC offers better resolution, a dirty sample will quickly degrade the analytical column. Disposable guard columns have been developed to protect the main HPLC column, but this configuration is in effect a parallel LC

system with a clean-up column and an analytical column in series. Initially the column material of the two columns were identical i.e. a 5 μm silica guard column protecting a 5 μm silica analytical column. However, once the functions of the two columns were separated i.e. the guards column was designated a clean-up column and not a disposable analytical column, it was clear that the clean-up column may be more effective with a different packing and function.

2.6 GROUP SEPARATION AND MULTI-RESIDUE SCHEMES

The power of analytical instrumentation currently available makes it possible to detect toxic organic contaminants at concentrations below 10^{-12} in environmental samples which can place additional demands on the sample extraction, clean-up and group separation schemes as more chemicals are added to the list of determinedness. Master Analytical Schemes [32,109–114] have been used to great effect for the determination of specific contaminants in water, e.g. EPA Priority Pollutants Protocols. However, the different types of sediment and biological matrices sometimes makes it difficult to devise and to validate a single analytical scheme for a wide range trace organic contaminants. The wide-angle analytical approach which adds additional compounds to a single scheme ultimately leads to a conflict of methodologies and an unacceptable level of compromise. As a result, two styles of sample preparation schemes exist. Firstly, the specific method, for the determination of a single or very small group of similar compounds, and secondly, multi-residue scheme which cover the preparation and separation of the maximum number of compounds possible for broad based chemical information. Examples of the range and style of group separation and multi-residue schemes for trace persistent organic contaminants are given in Table 2.7.

There are a number of related factors which have restricted the overall success of the separation of the persistent contaminant types into specific groups. The first is philosophical. Most of the effort of the analytical development has gone into isolating groups of similar compounds together in one eluate, i.e. all CBs in one group, all PAHs in another and all CHBs in a third. This scheme has an administrative simplicity and it also means that the analytical effort is less if the determinedness are grouped together and analysed in one chromatogram. However, where any one group of determinedness are measured as part of a multi-residue scheme, the need to have that one group of compounds in the same fraction diminishes.

The difficulty in separating compound types into groups is compounded by a number of factors. Many of the groups of compounds (e.g. CBs, CHBs, PAHs, PCNs) are in themselves complex mixtures covering a spectrum of physico-chemical properties which in themselves overlap significantly. Therefore, some of the between-group physico-chemical properties are less than the overall within-group properties. Most of the chromatographic techniques, hitherto, applied to group separation have utilised relatively low resolution and unlikely to provide the necessary *clean* separation which is required. The range of the chromatographic phases used for these separations has also been limited.

The overall environmental persistence of these contaminants is due to their stability to metabolism and microbial degradation and is reflected in the lack of functional groups on the molecule. This relative unreactivity reduces the range of chemical manipulations

TABLE 2.7

GROUP SEPARATION OR MULTI-RESIDUE SCHEMES FOR TRACE ORGANIC COMPOUNDS

Analyte groups	Matrix	Extraction	Clean-up	Group-Seperation	Reference
Adsorption columns for fractionation					
CBs, PAHs, phenols, chlorobenzenes, chloroanilines, etc.	Sediments and sewage sludges	Sonication and blending	GPC	Alumina	Kolb et al., 1995 [164]
Alkanes, PAHs, isoprenoids	Crude oil			Silica gel	Wang et al., 1994 [101]
Alkanes, PAHs, ketones, aldehydes, steroids, alcohols	Sediments	Sonication	Silica gel	Silica gel	Desideri et al., 1996 [115]
Nitro-PAHs, oxygenated compounds	Grilled sausages, smoked herring	Blending with acetone	Biobead SX-3	Silica gel	Larsson et al., 1988 [116]
Aliphatic hydrocarbons, PAHs, DDT-group and CBs	Sediments	MWAE	Copper wire	Florisil	Pastor et al., 1997 [50]
OCPs, CBs, PCDDs/Fs	Sediments	Soxhlet	KOH/silica + H_2SO_4/silica + activated charcoal/carbon/glass fibre online or offline final clean-up with H_2SO_4/silica + alumina in Pasteur pipette	See clean-up	Oehme et al., 1993 [165]
OCPs, PCBs, PCDD/Fs, PCNs	Salmon, eagle	Soxhlet	Sulphuric acid	Florisil, carbon, alumina	Tarhanen et al., 1989 [166]
CHBs, CBs, OCPs	Cod liver oil	LLE with DMF/hexane	Sulphuric acid	Silica gel	Lach and Parlar, 1991 [99]
CHBs	Fish muscle and narwhal blubber	Column percolation	Filter, GPC,	Florisil, (GC/MS-MS)	Chan et al., 1998 [98]
GPC or SEC-columns for fractionation					
PAHs, OCPs	Oysters	Reflux with potassium hydroxide	Partition into DCM, alumina column, MPLC	ZSM-5 zeolite SEC	Fisher et al., 1993 [94]
OCPs, PCBs, PCDDs, PAHs and thiophosphate insecticides	Environmental matrices	SFE	Graphitic carbon on glass fibres	GPC, Florisil, silica gel	Tilio et al., 1994 [141]

TABLE 2.7 (*continued*)

Analyte groups	Matrix	Extraction	Clean-up	Group-Seperation	Reference
PAHs, PCBs, OCPs and coprostanol	Sediment, mussel tissue, oyster tissue	Blending	Filter (sediments) or alumina/silica gel for biota	GPC	Krahn et al., 1989 [149]
PAHs, PCBs, OCPs	Mussel tissue (SRM 1974)	Soxhlet	Silica SPE Amino SPE	GPC, amino HPLC	Wise et al., 1991 [118]
HPLC-columns for fractionation					
CBs	Herring	KOH-ethanol, followed by hexane LLE		LC–GC online (CN-phase 100 × 2.1 mm × NB 54 15 m × 0.32 mm)	Hyvönen et al., 1991 [119]
Aliphatic hydrocarbons, PAHs	Sediment, clam, shrimp, sole	HCl/DCM (sediments), 3 cycles of MeOH/ DCM (biota)	Filter through glass wool plugs in funnels; silica sep-pak (sediments), florisil (biota)	Amino-HPLC, Waters	Morel et al., 1991 [117]
Aliphatics, monocyclic aromatics, PCBs, PCNs and PCDD/Fs Bandh et al., 1993 [167]	Environmental samples	Dean-Stark Soxhlet	Silica gel	2D-HPLC (nitrophenylpropylsilica- and 2-(1-	pyrenyl)ethyldimethylsilylated silica-column)
Musk xylene, musk ketone, musk ambrette, musk tibetene	Fish and blue mussels	LLE acetone water petrolether	GPC	Silica gel	Rimkus and Wolf, 1993 [168]
OCPs, PCBs, PCTs (terphenyls)	Fat-containing food and biota samples		GPC, silica gel	HPLC (hypersil Si 5 μm)	Rimkus et al., 1996 [91]
Nitrogen- and phosphorous containing pesticides	Marine sediment	Soxhlet	C_{18} cartridges	HP-GPC (Polygel 300 × 7.5 mm) or biobead SX-3	Bester and Hühnerfuss, 1997 [92]
PAHs, OCPs, PCBs, PCDD/Fs	Sediment, plankton	Dean-Stark Soxhlet	Dialysis	Florisil, HPLC (amino-column), HPLC (PX-21 carbon column)	Bergqvist et al., 1992 [169]
PAHs, PCBs, PCDD/Fs	Sediment, fish	Dean-Stark Soxhlet	Silica gel	2D-amino- and carbon-HPLC system	Zebühr et al., 1993 [100]
Isolation of a single group with or without fractionation					
PCTs	Fish lipids	Soxhlet	GPC	GPC (60–100 g SX-8 or SX-3)	Hale et al., 1991 [152]

PCDD/Fs	Environmental samples incl. biota		Review			Singh and Kulshrestha, 1997 [170], (review)
PCDD/Fs	Environmental samples incl. biota		Review			Ballschmiter and Bacher, 1996 [171] (review)
CHBs	Fish and sediment	Column percolation	GPC (SX-3)	Florisil		Donald et al., 1998 [34]
Alkylbenzenesulphonates	Sediment	Soxhlet	derivatisation and chromatography on alumina, silica and copper	SAX (carried out before clean-up)		Reiser et al., 1997 [24]
Alkylphenols, alkylbenzenesulphonates	Sediments	ASE	Sonication/filtration			Kreißelmeier and Dürbeck, 1996 [62]
Nonylphenols	Sewage sludge and sediment	Soxhlet	Alumina + derivatisation with pentafluorobenzylbromide			Chalaux et al., 1994 [23]
Bromocyclenes	Fish	LLE water-acetone-petrolether	GPC	silica gel		Bethan et al., 1997 [172]
OCPs	Sediments	Soxhlet	Florisil			Tan and Vijayaletchumy, 1994 [97]
Multiresidue schemes without fractionation						
CHBs	Fish, sediment	Blending with acetone/hexane after different treatments (acid, alkaline, nitration)				Calero et al., 1993 [33]
Base/neutral and acidic semi-volatile organic contaminants	Sediments	Sonication	Copper powder, SPE (C_{18}-cartridge)	(GC/ITMS)		Davis et al., 1993 [173]
PAHs, CBs, PCDD/Fs, phenols and unknowns	Marine sediments	Ultrasonication	GPC, H_2SO_4	(GC/MS or GC/AED)		Pedersen-Bjergaard et al., 1996 [174]
PAHs, OCPs, phenols and base/neutral compounds	Reference soils and sediments	MWAE	Silica gel	(combination of detectors)		Lopez-Avila et al., 1994 [47]

possible and, limits the separation methods based on functionality alone. Most separations have used either silica or florisil with varying degrees of activity, controlled by the moisture content of the adsorbent [98,99,101,115,116]. The separation of these groups of compounds has been based on polarity which result in the eluting compounds being distributed in different chromatographic fractions, e.g. some CBs and pp'DDE in one group, chlordanes and CHBs in another. Some of these separations have been further refined by using HPLC [91] and silica with bonded amino [100,117,118] and cyano [119] groups. The HP-GPC columns recently developed provide a substantial improvement over the low resolution GPC Biobead columns used mainly to separate the lipids [92] (Table 2.7).

One of the most significant group separations based on spatial configuration rather than polarity or size has been the success of the porous graphitic [120,121] carbon and the pyrenyl-silica HPLC columns [122–125] to separate contaminants on the basis of planarity.

Non-planar		Planar
Di/tri ortho CBs	Mono ortho CBs	Non ortho CBs
OCPs		PCDDs, PCDFs
CHBs and the brominated homologues		PCNs, PAHs
n-Alkanes		

The main difference between these groups of persistent contaminants is their spatial configuration, as demonstrated by the above example. Enzyme-Linked Immunosorbent Assay (ELISA) has been applied to the determination of PCBs in a kit for screening purposes [126]. The technique uses an anti-PCB antibody, stabilised, preserved and immobilised on paramagnetic particles, a PCB enzyme conjugate, e.g. horseradish peroxidase-labelled PCB analogue plus the sample extract. Normally the total PCB is analysed colorimetrically. The ELISA method itself is relatively insensitive, but the normal colorimetric detection can be replaced. The antibody-determinant conjugate can be isolated and hydrolysed, and the liberated group of CBs which are now separated from other contaminant can be measured with a more sensitive detector. The elegance of such a method is that the enzyme conjugate is spatially specific, which is exactly what is needed to isolate these contaminants for each other.

2.7 CONFIRMATORY METHODS

Viana et al. [127] used separate combinations of concentrated sulphuric acid (oxidative dehydration), potassium hydroxide (saponification) and chromium(iv) oxide on samples of OCPs and PCBs to identify compounds on the basis of the disappearance of after chemical attack. It is surprising that these older chemical methods are still in use when they are clearly inadequate for confirmatory purposes, particularly for polluted samples and when MS is used. The mass spectra provides a highly specific information on contaminants and, in conjunction with the high resolution GC retention index, provides unequivocal identification.

2.8 QUALITY ASSURANCE AND INTERLABORATORY STUDIES

Participation in External Quality Control schemes by laboratories who routinely under-take these analyses form an essential part of any quality assurance programme. When these programmes are coupled with practical workshops and development/training exer-cises then they become valuable aids to method development. They also provide a means whereby it is possible to establish groups of laboratories which can provide data of known quality which is fit for its intended purpose. Law et al. [128] reported on the laboratory performance studies for PAHs in sediments under the QUASIMEME programme. The robust coefficients of variation (CV%) for the three rounds ranges from 8–30%, 13–22% and 8.8–21%, respectively, for solutions and 17–34% for raw sediment extracts. De Boer and Wells [129] reported on similar studies in the same project for CBs and OCPs in fish and sediments over the 3-year period 1993–1996 with 5 separate laboratory studies. There were a number of laboratories that performed very well in these studies and there were also a number of participants whose performance improved considerably. However, at the end of that 3-year phase of the project there were still a number of determinedness which had CV% of >50% for all laboratories. Many laboratories still experienced difficulty in determining these contaminants at level around 1 μg kg^{-1}. Six USFDA laboratories participated in an interlaboratory trial using Mega Bond Elute C_{18} to determine the level of agreement for the clean-up steps in the determination of OCPs in non-fatty fish tissues. The mean recoveries ranged for 89 to 108% with a within-laboratory CV% of between 3.5% and 18%, and a between-laboratory agreement of 11% to 27% at the 0.04 mg/kg level [130].

2.9 HYPHENATED TECHNIQUES

One specific problem relating to the automation organic contaminant analysis is the effect of non-volatile co-extracted material on the performance of the GC when the sample is injected onto the high resolution analytical column. GPC is ideally suited to this separation on the basis of molecular size and can be used to provide an automated sample preparation where volatile contaminant require isolating prior to GC analysis. Blomberg et al. [131] developed an automated sample clean-up using on-line coupling of GPC to high resolution GC. The transfer technique used concurrent solvent evaporation using a loop interface, early vapour removal and co-solvent trapping. Compounds as volatile as n-tridecane were quantitatively trapped. The technique included compounds with a range of volatility from n-C_{12} to n-C_{60}. Blomberg et al. [132] further developed the automation of the on-line GPC with the addition of the normal phase LC (NPLC) prior to the high resolution GC. The first development with GPC coped with the separation of the high molecular weight materials, but not those co-extracts which interfere with the target compounds, many of which are low molecular weight semi-volatile materials themselves. This clean-up problem was overcome by using a NPLC as a second step. The potential incompatibility of the polar solvent from the GPC and the apolar NPLC solvent was resolved with a cryogenic cold trap to focus the GPC eluate which was subsequently injected into the LC column at ca. 50°C ramping at 1 deg.min^{-1} to 300°C. The LC was connected to the GC via a 30 cm × 50 μm i.d. silica needle. This configuration produces

3D chromatography in a very similar way to the modulated multi-dimensional GC (MDGC) where two GC capillary columns provide orthogonal separation in the gas phase [133–135]. The advantages of this method is that lipophilic materials that are not completely separated on the GPC are removed from the sample on the NPLC column, thus overcoming the problems associated with the clean-up of fatty tissues with GPC reported by Grob and Kälin regarding [136].

SPE systems have been interfaced to GC–MS [137] with a modified loop-type interface with small desorption volumes (50–100 μl in combination with a long 0.5 mm i.d. retention gap. Although the system was developed for the extraction of pesticides from water, the hyphenated construction can be used for extracts from other environmental matrices. The detection limit was ca. 0.1 μg kg^{-1} and 2–20 ng/kg with the MS in the SIM mode. The system was applied to several classes of contaminants including chlorophenols, chloroanilines, triazines, OPs and nitromusk compounds. An overview of the advantages and disadvantages of on-line LC–GC with the present state of the art are given in Table 2.8.

2.10 HEALTH AND SAFETY

In preparing this overview chapter it has become clear that the environmental chemist could be more diligent in developing methods that are more environmental friendly and provide a safer working environment. Following the Montreal Protocol there is a gradual

TABLE 2.8

ADVANTAGES AND DISADVANTAGES OF ON-LINE LC–GC WITH THE PRESENT STATE-OF-THE-ART TECHNOLOGY

Advantages	Disadvantages
Rapid, direct analysis with considerable saving in time	Potential loss of high resolution in coupling. Concurrent solvent evaporation needs very careful matching of conditions to have the same precise focusing as on-column injection of small volumes
Use of all the sample with considerable increase in sensitivity where appropriate or reduction in the initial sample volume	Limited to on-column injection techniques with a minimum of 80° difference in temperature between solvent and the first determinant
Reduction in the amount of solvent used, especially with narrow or microbore LC	Not possible to analyse multiple eluates from the LC. Multi-residue analysis difficult
Less chance of introducing an artefact in the sample.	Separation of interfering, co-eluting compounds not always possible
With less solvent there are lower levels of impurities	Present systems cannot cope with high lipid loading (ca. >500 mg)
Avoids loss of solute	Desulphurisation and large amounts of lipid to be removed 'off-line'
On-line group separation and sample preconcentration is possible	Memory effect can cause interferences. Lengthy optimisation and set time
Provides two independent sets of retention data for correlation to improve identification	*All* the sample is used in a single shot analysis. No chance to repeat analysis or determine other compounds in the extract

phasing out of the use of chlorinated solvents. Whilst this has the major environmental impact in the dry cleaning and bulk chemical industry, it seems inappropriate still to be developing methods that require chlorinated solvents such as DCM when, with a review of the literature and some application, suitable alternatives can be used. Some chemicals such as mercury in open systems and benzene as solvents should simply be banned from use in the laboratory in view of the known toxic effects of these chemicals. There should be a clear move to containerisation of methodology which utilises organic solvents. The welcome development of automated extraction with smaller solvent volumes is both safer and less costly.

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Chapter 3

Applications of microwave-assisted extraction in environmental analysis

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CONTENTS

3.1 SAMPLE PREPARATION TECHNIQUES IN ENVIRONMENTAL ANALYSIS

Sample preparation in environmental analysis is a lengthy process prone to errors, which has gone through quite a transformation in the past decade. The Soxhlet extraction technique, which has been so popular up until the mid 1980s, is now being replaced by supercritical fluid extraction (SFE), microwave-assisted extraction (MAE) techniques and accelerated-solvent extraction (ASE). Over the years analytical laboratories must have disposed of hundreds of thousands of liters of organic solvents, either as solvent waste or as vapors emitted to the atmosphere. The most significant drawbacks of Soxhlet extraction are long extraction times, lack of sample agitation, and a mandatory solvent evaporation/concentration step after extraction. Attempts made to automate the technique were somewhat successful and a few commercial systems (e.g., Soxtec of Tecator/Perstorp Analytical and Soxtherm of O.I. Analytical) are available.

Table 3.1 summarizes the most common extraction techniques for solid matrices. Conventional Soxhlet extraction will continue to be used most likely in regulatory work

TABLE 3.1

EXTRACTION TECHNIQUES USED IN SAMPLE PREPARATION FOR ENVIRONMENTAL ANALYSIS[a]

Extraction technique	Principles	Approved US EPA Method
Conventional Soxhlet	Sample is placed in an extraction thimble and leached with hot solvent in a Soxhlet extractor for 8–12 h. Solvent evaporation/ concentration is done separately	3540
Automated Soxhlet	Sample is placed in an extraction thimble and immersed in boiling solvent for 30–60 min; thimble is then raised for Soxhlet extraction with solvent refluxing. Solvent evaporation/concentration is possible	3541
Supercritical fluid extraction	Sample is placed in a high pressure cartridge or chamber and extracted with supercritical fluid (e.g. carbon dioxide at pressures of 150–450 atm and temperatures of 40–150°C). After depressurization, analytes are collected in a small volume of organic solvent or on a trap	3560, 3561, 3562
Microwave-assisted extraction	Sample is placed in an open or closed vessel, immersed in solvent and heated with microwave energy	3546
Accelerated solvent extraction	Sample is placed in extraction vessel and pressurized with solvent heated above its boiling point; the extract is automatically removed and transferred to a vial	3545

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because it is rather inexpensive and is the reference method by which many of the newer extraction techniques (e.g., SFE, MAE, and ASE) are evaluated. The SFE technique, for example, has gained an important place among sample preparation techniques for solid matrices because (a) it uses a chemically inert, non-toxic, non-flammable solvent (e.g. carbon dioxide) for extraction; (b) the extraction selectivity can be controlled by varying the pressure and temperature of the supercritical fluid and by addition of modifiers; (c) it provides for easy removal and disposal of the extraction solvent; and (d) the SFE systems that are available commercially allow sample extraction in unattended operation (up to 44 samples can be extracted sequentially). More recently, the MAE technique has been investigated because it offers (a) greatly reduced volumes of organic solvents (30 ml for MAE versus 300 ml for Soxhlet or sonication extraction), (b) reduced extraction time (typical sample preparation for MAE is 10 min for extraction and 40 min for pre- and post-extraction sample handling), (c) increased sample throughput by the use of multivessel systems that allow simultaneous MAE of multiple samples (d) precise control of temperature during the extraction, and (e) ease of use and not labor intensive.

Despite their advantages, SFE and MAE techniques are not widely used in analytical

laboratories performing regulations-driven analytical work, because they have not been promulgated by the US Environmental Protection Agency (EPA). Three SFE methods (e.g. Method 3560 for the extraction of petroleum hydrocarbons from soils, Method 3561 for the extraction of polynuclear aromatic hydrocarbons from solid matrices, and Method 3562 for the extraction of organochlorine pesticides) are the only SFE methods that have received the approval of the EPA Solid Waste Organic Methods Workgroup and are expected to be included in future updates of the EPA's Office of Solid Waste (OSW) SW-846 manual. A draft protocol for MAE was submitted recently to the EPA OSW for review, but the official approval of this method is probably going to take at least 1 year. ASE has been approved by the EPA.

The purpose of this chapter is to discuss the principles of MAE and to summarize the developments in MAE technology with primary focus on environmental applications. For further information on the fundamentals of microwave-assisted chemistry the reader should refer to a book by Kingston and Haswell published by the American Chemical Society in 1997 [1].

3.2 FUNDAMENTALS OF MAE

3.2.1 How microwaves work

Microwaves are high-frequency electromagnetic waves placed between radio frequency and the infrared regions of the electromagnetic spectrum (their frequencies range from 0.3 to 300 GHz). They are used for cooking foods but recent industrial applications of microwaves include materials processing, soil remediation, and organic synthesis.

In contrast to conventional heating where the heat penetrates slowly from the outside to the inside of an object, microwave energy is ''cold'' producing heat (heating takes place by dielectric loss). Therefore, the heating appears right in the core of the body that is being heated, and the heat spreads from the inside to the outside of that body. The microwave energy affects molecules by ionic conduction and dipole rotation. In ionic conduction, the ions in solution will migrate when an electromagnetic field is applied. The resistance of solution to this flow of ions will result in friction and, thus, heating of the solution. Dipole rotation means realignment of the dipoles with the applied field. At 2450 MHz, the dipoles align and randomize 4.9×10^9 times per second; this forced molecular movement results in molecular ''friction'' and, thus, heating of the solution [1].

3.2.2 Solvent selection

Selection of proper solvent is the key to a successful extraction. In selecting solvents consideration should be given to the microwave-absorbing properties of the solvent, the interaction of the solvent with the matrix, and the analyte solubility in the solvent (the principle of ''like dissolves like'' is still applicable in the MAE). The larger the dipole moment of the solvent the faster the solvent will heat under microwave irradiation. For example, hexane (dipole moment is <0.1 Debye) will not heat whereas acetone with a dipole moment of 2.69 Debye will heat in a matter of seconds. Thus, a mixture of hexane–acetone is an ideal solvent for compounds of environmental significance and many applications described here use hexane–acetone (1:1).

Other important factors under consideration include: (a) the compatibility between the

extraction solvent and the analytical method used in the analysis of the extract (the less polar solvents seem to be preferred for gas chromatographic analysis, whereas the more polar ones for liquid chromatographic analysis and immunoassay techniques) and (b) the selectivity of the solvent. Little has been reported in the literature on the selectivity of MAE because the technique is so efficient that it could not be regarded as a selective extraction technique. ''Everything gets extracted'' so a clean-up step after the extraction is needed in almost all cases.

When MAE is conducted in closed vessels, the temperature achieved during the extraction will be greater than the boiling points of the solvents. Table 3.2 lists the boiling point and the closed-vessel temperature at 175 psi for several solvents and solvent mixtures. For most of the solvents the temperature inside the vessel is two to three times the boiling point of the solvent. These elevated temperatures result in improved extraction efficiencies of the analyte from the sample matrix.

The most common solvents for environmental applications are hexane–acetone [7–12, 29,30,34,37,39,51,53,54,60,61,77,79,89,91] and dichloromethane [22,23,35,45,50–52,61, 95], but other solvents including non-polar solvents such as hexane [31,68] and isooctane [18,42], chlorinated solvents such as carbon tetrachloride [57], or more polar solvents such as acetone [22,43,50,95], methanol [3,18,96], acetonitrile [5], water [18,73], 2 M sodium hydroxide [21], and ammonium hydroxide/ammonium acetate [25,36,38] have been reported. Among the binary mixtures, chloroform-methanol [44], methanol-water [3,4], dichloromethane–toluene [77], isooctane–acetonitrile [5], methanol–toluene [53], dichloromethane–water, methanol, or acetonitrile [40,63], and acetone–petroleum ether [51–54] have been reported. A ternary mixture of hexane-dichloromethane-ethyl acetate was reported in Ref. [19].

TABLE 3.2

SOLVENT BOILING POINT AND CLOSED VESSEL TEMPERATURES[a]

Solvent	Boiling point (°C)	Closed vessel temperature (°C) at 175 psi
Dichloromethane	39.8	140
Acetone	56.2	164
Methanol	64.7	151
Hexane	68.7	
Ethanol	78.3	164
Cyclohexane	80.7	
Acetonitrile	81.6	194
2-Propanol	82.4	145
Petroleum ether	35–52	
Acetone-hexane (1:1)	52+	156
Acetone-cyclohexane (70:30)	52+	160
Acetone-petroleum ether (1:1)	39+	147

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Food matrices have been extracted with very polar solvents such as water [73], methanol [3], methanol–water [3,4], 2-propanol–acetonitrile [76], and 2-propanol–petroleum ether [71]. Marine tissues have been extracted with dichloromethane [24], dichloromethane–toluene [24], acetone–hexane [24], ethyl acetate–cyclohexane [26,80], ethanol–propanol [45,59] and *n*-hexane [55].

Solvents used to extract additives from the polymers (e.g. polyethylene, polypropylene) include alcohols (e.g. 2-propanol, isopropanol, isobutanol), acetone [81], chlorinated solvents such as 1,1,1-trichloroethane [6] and chloroform [28], and mixtures of alcohols and chlorinated solvents such as 2-propanol–cyclohexane [81]. Since polymers swell in boiling solvents, a solvent-to-polymer ratio of 10:1 is needed for efficient extraction. Acetone–cyclohexane (7:3) has also been used but is less desirable because reaches pressures in excess of about 6 atm at 140°C whereas 2-propanol and isobutanol reach only 5 and 2 atm, respectively, at 140°C [1].

3.2.3 Temperature and moisture effects

In principle, elevated temperature increases the analyte solubility in the solvent and weakens the adsorption bonds between the analyte and the matrix. However, when dealing with environmental samples containing more than one analyte, the actual data may indicate the contrary because some compounds may be thermally labile and others may catalytically decompose in the presence of soil. Figs. 3.1 and 3.2 show recoveries of a group of 47 organophosphorus compounds and 44 organochlorine pesticides, respectively, as a function of temperature, matrix and solvent type. Details of the data can be found in Ref. [70]. It is quite evident from the two figures that all recoveries were lower at 145 than at 50°C. The explanation lies in the fact that several organophosphorus pesticides were thermally unstable at 145°C. For example, TEPP (tetraethyl pyrophosphate) is known to decompose at 170°C. MAE data indicated that this compound is stable when heated up to 145°C in solvent alone (recovery 102 and 95.8%). MAE recoveries dropped to 14.2% when dry soil was present and were even lower when wet soil was extracted by MAE [70]. Other examples include trichlorfon and naled, which are converted to dichlorvos by dehydrochlorination and dehydro-bromination, respectively. This reaction appears to take place during MAE even in the absence of soil. For example, when using hexane–acetone, trichlorfon recoveries were 86% at 50°C (5 min heating) and 79.1% at 145°C (5 min heating) but they dropped to 32% at 145°C (20 min heating). For solvent/dry soil suspensions, MAE recoveries were 11.9% (145°C/ 5 min) and 8.9% (145°C/20 min). In the case of naled, recoveries dropped from 90.1 to 23.5% when going from 50°C/5 min to 145°C/5 min and no naled was detected in extracts from 145°C/20 min experiments [70].

Monocrotophos, demeton-O, and demeton-S also showed consistently lower recoveries when heated in the solvent/dry soil and solvent/wet soil suspensions and their recoveries appeared to be a function of temperature and heating time [70].

From the group of 44 organochlorine pesticides investigated by Lopez-Avila et al. [70] only two compounds had recoveries below 10%. They were: hexachlorocyclohexane, a fairly volatile compound, which may have been lost during evaporation of extracts, and dichlone.

In another study, Lopez-Avila et al. [69] reported the effect of temperature on 95 semi-

Fig. 3.1. Effect of temperature, matrix, and solvent on the MAE recoveries of organophosphorous pesticides. From Ref. [70] with permission.

volatile organics (e.g. 34 neutral compounds including chlorinated aromatic compounds, nitroaromatic compounds, phthalate esters, 22 PAHs, 19 basic compounds, 19 phenols, and benzoic acid). Only some of the basic compounds (e.g. benzidine, α,α-dimethylphenethylamine) were most affected by temperature.

Quite often the effects of the various parameters affecting MAE recoveries (e.g. temperature and solvent) have to be addressed together. Llompart et al. [62] generated
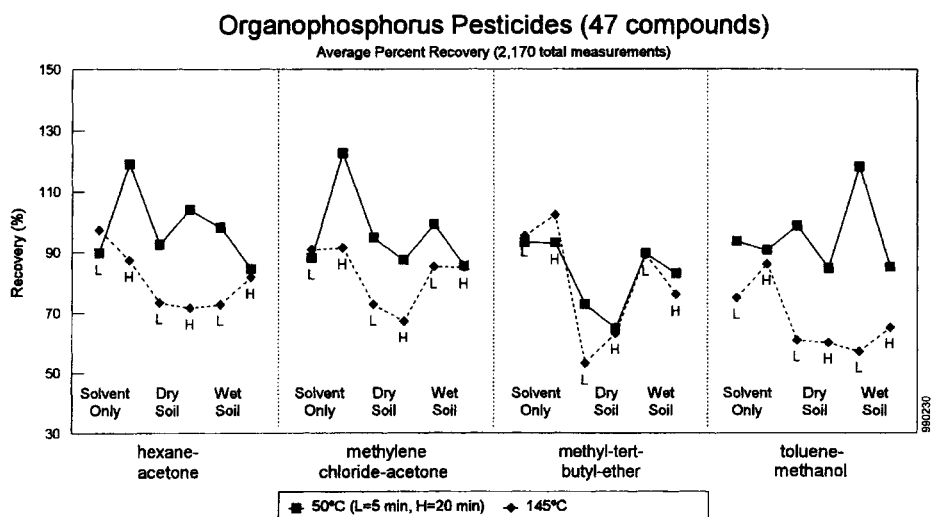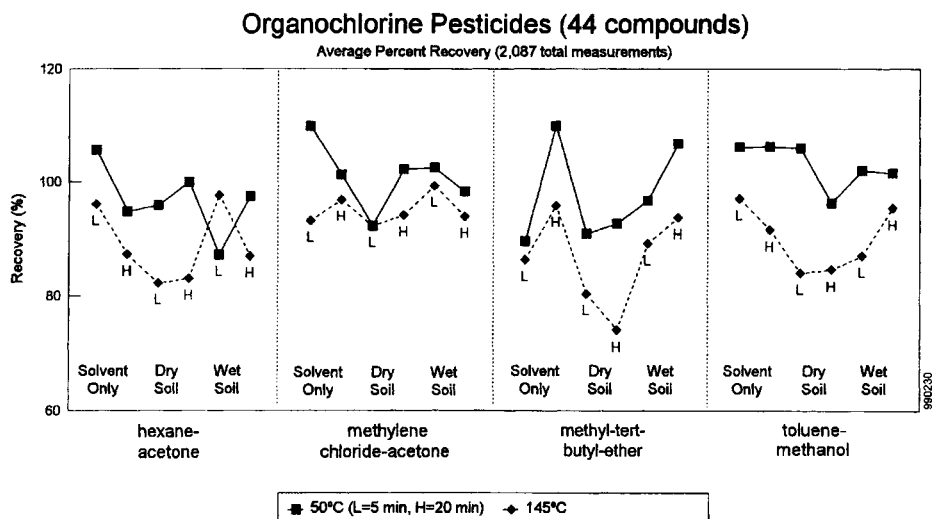

Fig. 3.2. Effect of temperatures, matrix, and solvent on the MAE recoveries of organochlorine pesticides. From Ref. [70] with permission.

response surfaces for phenol and methyl phenols to understand the effects of temperature, volume of solvent, and the composition of the extraction solvent and reported that extraction efficiency was directly proportional to temperature and the percentage of acetone in hexane for phenol and p-cresol. In the case of m-cresol, however, the response presented a maximum between 120 and 130°C when the percentage of acetone was at its highest level (e.g. 80%).

When dealing with environmental samples, most of them contain water ranging anywhere from 10 to 15% in the dry ones to 40% in the clay soils or sediments. The water present in the matrix allows local heating and this can accelerate the extraction process [77]. Several researchers [5,7,8,60] have reported on the effect of water in closed vessels and have attributed the increase in extraction efficiency to interactions between water and microwaves. Budzinki et al. [77] reported that addition of water to bring the moisture content of soils/sediments to 30% is needed in order to get reproducible and quantitative recoveries of PAHs in an open-vessel microwave system.

3.3 INSTRUMENTATION

3.3.1 MAE system components

The equipment (Fig. 3.3) used for MAE consists of a magnetron tube, an oven where the individual extraction vessels (closed vessels) are set up on a turntable or rotor, monitoring devices for temperature and pressure, and electronic components. It usually includes specific safety features such as rupture membranes for the extraction vessels, an exhaust fan to evacuate air from the instrument cavity, a solvent vapor detector (monitors the presence of solvent vapor in the microwave cavity and shuts off the microwave energy whenever solvent vapor is detected in the instrument cavity), an expansion container (the
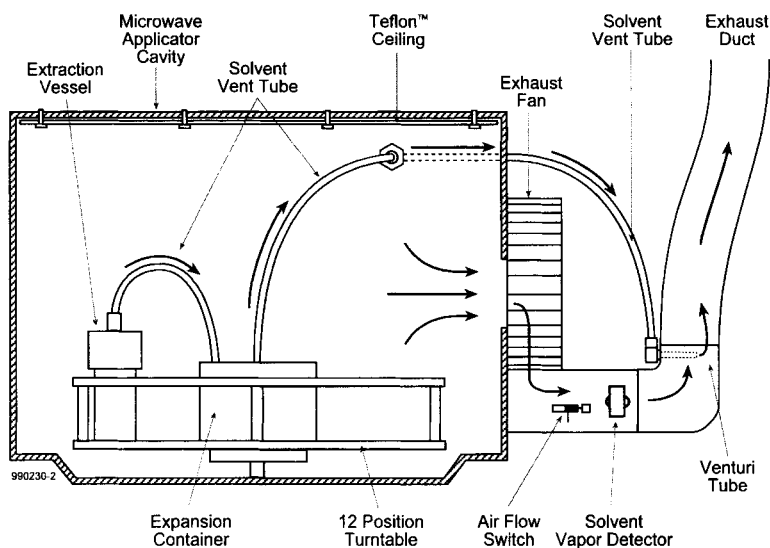

Fig. 3.3. Schematic diagram of the CEM MAE closed-vessel system. Reprinted with permission from V. Lopez-Avila, *Crit. Rev. Anal. Chem.*, 29 (1999) 195.

extraction vessels are connected to this expansion container through vent tubing; in case the membrane ruptures, due to increased pressure in the vessel, then vapor is removed through the rupture vent tube), and an isolator located in the wave guide that diverts reflected microwave energy into a dummy load to reduce the microwave energy within the cavity. One manufacturer of microwave equipment uses resealable vessels. In this case, vessels are placed on a sample rotor and secured with a calibrated torque wrench for uniform pressure. If the pressure exceeds the vessel limits, a spring device (Milestone's patented technology) allows the vessel to open and close quickly, thus releasing the excess pressure. These sample rotors are available with PFA and TFM liners with pressure ratings of 435–1450 psi. Another safety feature which was added to the microwave system is the ''movable wall''. To prevent the door from being blown away, a door frame on spring-loaded high-impact steel bars was added such that the door moves out and in to release pressure from the microwave cavity.

Typical pressures reached with most closed-vessel systems (first generation) was 7 atm but today's technology can handle pressures as high as 100–110 atm. A special rotor, which houses six thick-walled vessels capable of working at 110 atm, is available commercially on several systems including the CEM's MARS-5, Milestone's Ethos-1600, and Plazmatronika's UniClever system. In the Milestone system for example, if the operating pressure inside the vessel exceeds the vessel limits, a special spring device will allow the vessel to open and close, thus reducing the pressure.

3.3.2 Vessel design and materials

The vessels are typically made of microwave transparent materials (e.g. polyether imide, or TFM (tetrafluoromethoxyl) polymer) and are lined with perfluoroalkoxy or Teflon™ liners. A new microwave system introduced recently by one manufacturer uses magnetic stir bars and allows extraction with polar and non-polar solvents, while agitating the sample and solvent to achieve efficient mixing and improve analyte recoveries. Fig. 3.4 shows a schematic of the CEM's lined digestion vessel with and without temperature and pressure control. Vessel body and cap are made of Ultem, a polyetherimide. The cap and cover of the control vessel are modified to allow a pressure sensing tube and a fiber-optic temperature probe. The fiber-optic probe is microwave transparent and is positioned in the control vessel using a glass thermal well. Infrared temperature sensors are also used to monitor the temperature inside the vessel. As the turntable revolves, the infrared sensor measures the temperature of each vessel. More detail on pressure and temperature feedback control can be found elsewhere [1].

Additional features such as magnetic stirring of the extraction solvent inside multiple sample vessels is possible at least on one commercial system (Ethos 1600 Labstation from Milestone, Inc.). Moreover, non-polar solvent such as hexane can now be heated at elevated temperatures by use of magnetic stir bars made of Milestone's proprietary Fluoropolymer Weflon (this polymer absorbs the microwave energy and subsequently transfers heat to the surrounding medium).

All closed-vessel systems that are available commercially are multivessel systems by evenly spacing the vessels on a carousel or rotor and rotating them through a pattern of a 360° oscillating turntable. Fig. 3.5 shows a schematic of the open-vessel system.
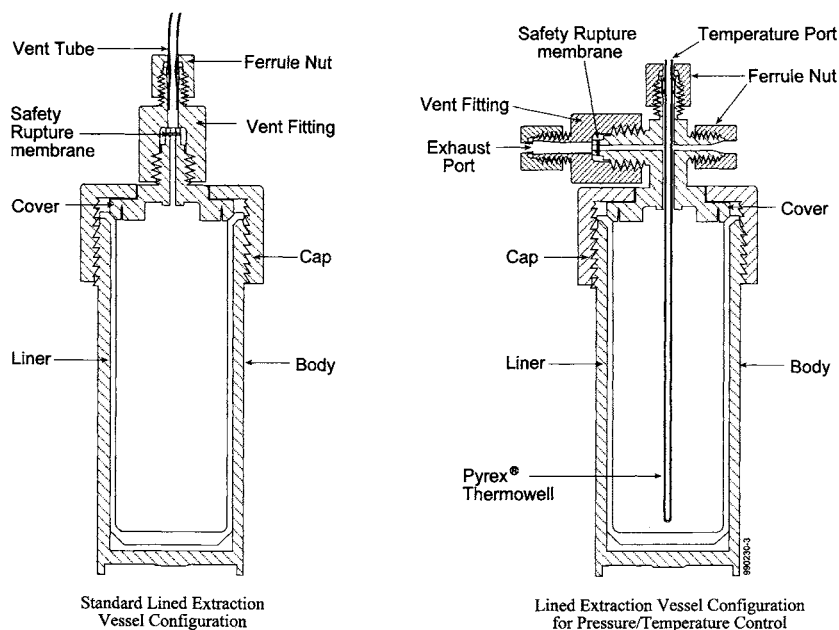
Fig. 3.4. Standard lined extraction vessel and lined extraction vessel with pressure and temperature control. Reprinted with permission from V. Lopez-Avila, *Crit. Rev. Anal. Chem.*, 29 (1999) 195.
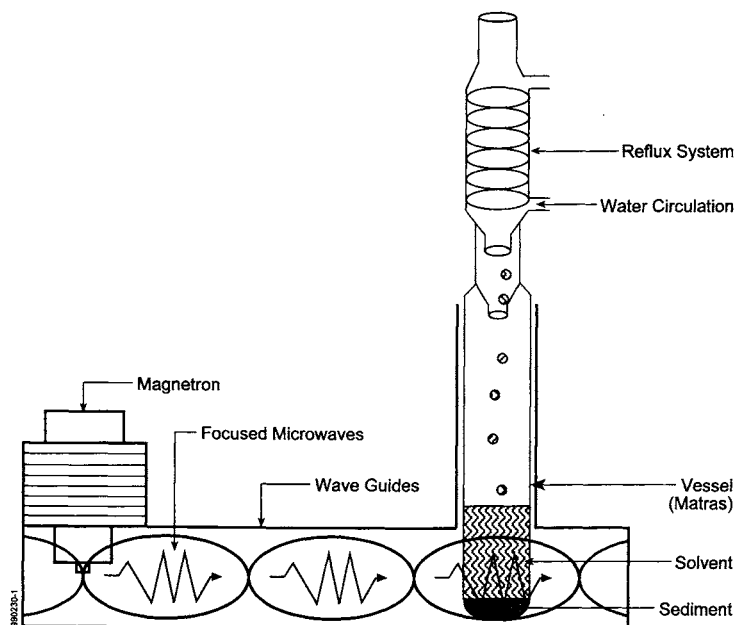
Fig. 3.5. Schematic diagram of the focused-microwave system from Prolabo (open vessel). Reprinted with permission from V. Lopez-Avila, *Crit. Rev. Anal. Chem.*, 29 (1999) 195.

TABLE 3.3

FEATURES OF COMMERCIALLY AVAILABLE MAE SYSTEM[a]

Model/manufacturer	Power (W)	Sensors	Max. pressure (bar)	Vessel volume (ml)	Vessel material	Number of vessels	Max. temp. (°C)
Multiwave/Anton Paar GmbH, Austria	1000	Pressure control in all vessels Infrared temperature measurement in all vessels	70 70 130 130 130	100 100 50 50 20	TFM/ceramics TFM/ceramics TFM/ceramics Quartz Quartz	12 6 6 6 6	230 260 260 300 300
MARS-5/CEM, USA	1500	Infrared temperature measurement in all vessels	35 100	100 100	TFM TFM	14 12	300 300
Ethos 900/1600, Milestone, USA	1600	Pressure control in all vessels Temperature control in all vessels	30 100 30 100	120 120 120 120	TFM or PFA TFM TFM or PFA TFM	10 6 12 10	240 280 240 280
Model 7195/O.I. Corp., USA	950		13 40	90 90	TFM TFM	12 12	200 200
Soxwave 100/3.6, Prolabo, France	250	Temperature control	Open vessel Open vessel	250 100 or 250	Quartz Quartz	1 6	

[a]Adapted from Ref. [2]. Reprinted with permission from V. Lopez-Avila, *Crit. Rev. Anal. Chem.*, 29 (1999) 195.

3.3.3 Commercial MAE systems

A product review on commercially available microwave systems was published recently in *Analytical Chemistry* [2]. In principle, there are three different type of units: closed vessel, open vessel, and flow-through systems, which allow both digestion and sample extraction. The special features of such systems are summarized in Table 3.3. The flow-through systems are not used in the extraction with flammable solvents and, therefore, will not be discussed here.

3.4 APPLICATIONS OF MICROWAVE-ASSISTED EXTRACTION

The microwave-assisted extraction (MAE) technology also known as microwave-assisted solvent extraction (MASE) was first reported by Ganzler and Salgo in 1986 [3]. Since then a number of applications, which are summarized in Table 3.4, were reported [3–101]. Specific target analytes reported in these applications include parathion bromophos, gossypol, vicine, and convicine [3,4], selected organochlorine pesticides or OCPs [5,7–9,11,29,30,54,55,60,80,85,89], polynuclear aromatic hydrocarbons or PAHs [7,8,11, 22–24,30,34,35,37,43,45,50,53,58,77,78,95], phenolic compounds [7,8,11,32,39,52,64, 91], polychlorinated biphenyls or PCBs [10,26,42,55,74,82], petroleum hydrocarbons [12,74], organotin compounds [18,46,72,99], organomercury compounds [67,99], trialkyl and triaryl phosphates [19], ergosterol [21], and imidazoline herbicides [25,36,38], antioxidants such as Irganox 1010, Irgaphos 168, and Chimassorb 81 [6,81] or Tinuvin 622 and Tinuvin 770 [28]. Other compounds extracted by MAE include: triazine herbicides [40,63,83,90], chlorinated benzenes [7,8,11,39,41], fatty acids [44], salinomycin [49,59], organophosphorus pesticides [50,70], phthalate esters [7,8,11,39,50,51], pyrones [73], terpenic compounds [75,97], chloramphenicol [76], dimethomorph [88], amino acids [92], sulfamethazine [93], hexaconazole [94], L-hydroxyproline [98], and taxanes [96]. The matrices that have been extracted by MAE include soils, sediments as well as more unusual ones such as cottonseeds, fava beans, yeast, maize, walnut, fungal spores, mushrooms, and polymers (Table 3.4). Extraction of essential oils and other oils from biological materials such as plant and fish tissue by exposure to microwave energy was patented by Environment Canada [101]; in this patent, extraction of natural products from mint, sea parsley, cedar foliage, and garlic with hexane, methylene chloride, or ethanol in two or more stages is described.

3.4.1 Specific applications

PAHs: Work done by Lopez-Avila et al. [7] indicated that PAHs, with the exception of more volatile compounds such as naphthalene, can be extracted quantitatively (recovery >80%) from soil and sediment matrices with hexane–acetone (1:1) at temperatures of 115°C. Typical extraction times for batches of up to 12 samples (5 g each) are 10 min at 100% power (1000 Watts). This is the time of extraction from the moment the temperature inside the vessel reaches the set temperature. The maximum temperature that can be reached in closed vessels with hexane–acetone (1:1), as solvent, is 155–160°C [7]. The lower recoveries of naphthalene, acenaphthene, and acenaphthylene were attributed to the presence of water in the soil matrix (to prepare a representative aged soil sample, water

TABLE 3.4

APPLICATIONS OF MICROWAVE-ASSISTED EXTRACTION REPORTED IN THE LITERATURE

Analyte	Matrix	Solvent	MAE conditions	Reference
Parathion, bromophos	Soil	Methanol	30 s irradiation in open vessel; repeat up to seven times	[3]
Gossypol	Cottonseeds	Methanol–water (85:15)		
Vicine, convicine	Fava beans	Methanol–water (1:1)		
Fat	Yeast	Hexane		
	Maize			
	Soya bean			
	Cottonseed			
	Walnut			
	Meat flour			
Pyrimidine–glucosides, gossypol	Fava beans	Methanol–water (50:50)	30 s irradiation in open vessel; repeat three times	[4]
	Cottonseed meal	Methanol–water (50:50)		
Organochlorine pesticides	Sediment saturated with distilled water (1 g sample and 2 ml water)	Acetonitrile Isooctane Isooctane–acetonitrile (1:1)	30 s irradiation in open vessel; repeat up to five times	[5]
Irganox 1010, Irgaphos 168, Chimassorb 81	Polyethylene Polypropylene	1,1,1-Trichloroethane n-Heptane–acetone (1:1)	3–6 min irradiation in closed vessel	[6]
17 PAHs, 14 phenols, 20 organochlorine pesticides	Three reference marine sediments	Hexane–acetone (1:1)	Closed-vessel extraction at 80, 115, 145°C for 5, 10, 20 min	[7,8,11,39]

13 miscellaneous compounds (e.g. chlorinated benzenes, nitroaromatic compounds and phthalate esters)	Three reference soils Topsoil			
16 Phenols, 20 organochlorine pesticides	Topsoil Clay soil Sand Reference soil	Hexane–acetone (1:1)	Closed-vessel extraction at 115°C for 10 min	[9]
PCBs	Topsoil Claysoil Sand One reference soil Two reference marine sediments 24 Soils	Hexane–acetone (1:1)	Closed-vessel extraction at 115°C for 10 min	[10]
Petroleum hydrocarbons	Reference soil	Hexane–acetone (1:1)	Closed-vessel extraction at 150°C for 5–30 min	[12]
Atrazine and degradation products	Lupin seeds Rat feces	Water followed by 0.35 N HCl	Closed vessel, 95–98°C	[14]
Lupin alkaloid (Sparteine)	Lupin seeds Rat feces	Methanol–acetic acid (99:1) Methanol–water–acetic acid (50:47.5:2.5) Methanol Methanol–water–ammonia (50:48.5:1.5)	30 s irradiation, open vessel	[15]

TABLE 3.4 (*continued*)

Analyte	Matrix	Solvent	MAE conditions	Reference
Organotin compounds (mono-, di- and tributyltin; mono-, di-, and triphenyltin)	Two reference sediments	50% acetic acid Isooctane Methanol Water Artificial sea water	1–7 min irradiation in open vessel, up to 160 watts	[18]
Trialkyl and triaryl phosphates	Sediment	Hexane-dichloromethane-ethyl acetate (1:1:1)	9 s irradiation in open vessel; repeat up to 10 times	[19]
Ergosterol	Fungal spores	Methanol–2 M sodium hydroxide (5:1)	35 s irradiation in closed vessel	[21]
	Mushrooms Filtered air Artificially contaminated corn, grain dust, soil			
PAHs	Soil	Acetone Dichloromethane	20 min at 120°C in closed vessel	[22]
PAHs	Marine sediments	Dichloromethane	5–40 min irradiation at 30–90 W in open vessel	[24]
PAHs	Marine sediments	Dichloromethane	10 min irradiation at 30 W in open vessel	[24]
	Mussel tissue	Dichloromethane–toluene (50:50)		
	Air particulates	Acetone–hexane (50:50)		

Imidazolinone herbicides	Soil	0.1 M Ammoniumacetate/ ammonium hydroxide (pH 9–10)	3–10 min irradiation at 125°C in closed vessel	[25,36,38]
Irganox 1076 Irganox 1010 BHT Irgafos 168 Tinuvin 622 Tinuvin 770 Chimassorb 944	Polyethylene Polypropylene	Cyclohexane–chloroform– triethylamine (45:45:10)	15 min at 140, 150, 160, 170 and 180°C in closed vessel	[28]
Chlorinated pesticides	Soil	Acetone–hexane (1:1) Acetone–hexane (3:2)	15–20 min at 90–120°C in closed vessel	[29]
Eight base/neutral and acid compounds 13 PAHs 12 organochlorine pesticide	Four reference marine sediments One soil Three lake sediments	Hexane–acetone (1:1)	10 min at 115°C in closed vessel	[30]
Phenol Methyl phenols	Soils	Hexane and hexane–acetone (2:8) with pyridine and acetic anhydride for in-situ derivatization	130°C in closed vessel	[32,64]
PAHs	Reference marine sediments	Hexane–acetone (1:1)	5 min at 115°C in closed vessel	[34]
PAHs	Reference marine sediments	Dichloromethane	5–10 min at 35°C in open vessel	[35,45]
PAHs	Fly ash	Hexane–acetone (90:10)	70°C in closed vessel	[37]

TABLE 3.4 (*continued*)

Analyte	Matrix	Solvent	MAE conditions	Reference
Atrazine Simazine Metazachlor Desisopropylatrazine Desethylatrazine	Sand Peat Clay	Dichloromethane with water, methanol, and acetonitrile	5–45 min at 30–130°C	[40,63]
Chlorinated benzenes	Water	Helium purge	7 min	[41]
PCBs	Water	Extraction with isooctane	2 min at 70°C, closed vessel	[42]
PAHs	Soil	Acetone	20 min at 120°C, closed vessel	[43]
Fatty acids Total lipids Triacylglycerol Phospholipids Cholesterol	Animal food Powdered rat	Chloroform–methanol (2:1) Methanol followed by chloroform	2 min, open vessel	[44]
Organotin compounds	Sediments	0.5 M ethanoic acid in methanol	3 min, open vessel	[46]
Salinomycin	Chicken tissue (kidney, liver, muscle, ovarian yolk, fat)	Ethanol/propanol (15:2)	9 s	[49,59]
16 PAHs	Water samples preconcentrated on C_{18} membrane disks	Acetone	1, 3, 5, 10 min at 80, 100 and 120°C, closed vessel	[50]

19 Organochlorine pesticides Four aroclors Six phthalate esters Seven organophosphorus pesticides Five fungicides/herbicides		Dichloromethane		
Phthalate esters	Marine sediment Soil	Dichloromethane Acetone–hexane (1:1) Acetone–petroleum ether (1:1)	5, 10, 15 min at 80, 115 and 145°C, closed vessel	[51]
Nonyl phenol	Water samples preconcentrated on C_{18}-packed cartridge, C_{18} packed disk Sediments	Dichloromethane Acetone–petroleum ether (1:1)	5 and 15 min at 100–120°C, closed vessel	[52]
PAHs	Marine sediments	Dichloromethane Acetone–hexane (1:1) Acetone–petroleum ether (1:1) Methanol–toluene (9:1)	5 and 15 min at 115 and 135°C, closed vessel	[53]
Organochlorine pesticides	Water samples preconcentrated on C_{18} disks Marine sediments	Dichloromethane Hexane–acetone (1:1) Acetone–light petroleum (1:1)	5, 10, 20 min at 80, 100 and 120°C, closed vessel	[54]
PCB 153 PCB 180 PCB 138 p,p'-DDE	Seal blubber Pork fat Cod liver Water	n-Hexane Ethyl acetate-cyclo-hexane (1:1) Carbon tetrachloride	Several 30-s extractions at 1000 W Several irradiations at 250–1000 W in increments of 100 W	[55] [87]

TABLE 3.4 (*continued*)

Analyte	Matrix	Solvent	MAE conditions	Reference
Hexachlorocyclohexane Hexachlorobenzene Oil and grease			6 min at 420 W	[57]
PAHs	Polyurethane foam	Methanol followed by cyclohexane	Five times for 20 s (methanol) and five times for 25 s (cyclohexane)	[58]
29 chlorinated pesticides	Soil	Hexane–acetone (1:1)	15 min at 115°C, closed vessel	[60]
Low-molecular weight oligomers	Polyethylene terephthalate film	Hexane–acetone Water Acetone Acetone–dichloromethane Dichloromethane	30, 45, and 120 min at 115 or 120°C, closed vessel	[61]
Methylmercury	Aquatic sediments Certified reference sediments	Digestion with 6 M HCl (methylmercury is extracted at room temperature by complexation with cysteine acetate and toluene)	10 min at 120°C, closed vessel	[67]
Fat	Meat Dairy Egg products	Petroleum ether Hexane	60 s at 300 W, then 30 s off, followed by 90 s at 300 W	[68]
Chlorothalonil Azinphos-methyl	Beets Cucumbers	2-Propanol-petroleum ether (1:2)	10 or 20 min at 80, 100 and 120°C, closed vessel	[71]

Dacthal Methoxychlor Permethrin Diazinon Chlorpyrifos	Lettuce Peppers Tomatoes			
Butyl and phenyl organotin	Reference marine biological matrix Tuna tissue Mussel tissue	25% Tetramethyl- ammonium hydroxide in water	3 min at 90°C, 115°C, and 130°C, closed vessel	[72]
2-Methyl-3-hydroxy- 4-pyrone (Veltol) 2-Ethyl-3-hydroxy- 4-pyrone (Veltol-Plus)	Potato chips Corn	Water	10 min at 100°C, closed vessel	[73]
C_{16}–C_{32} hydrocarbons 20 PAHs Four organochlorine pesticides PCBs	Marine sediments	Toluene-water (1:5–1:2)	6 min, closed vessel	[74]
Terpenic compounds (Linalool, α-Terpineol, Citronellol, Nerol, Geraniol)	Vitis vinifera must preconcentrated on Amberlite-XAD	Acetone-dichloromethane (1:1)	80 and 120°C, 5–15 min, closed vessel	[75,97]
Chloramphenicol	Egg albumen Yolk	Dichloromethane Acetonitrile-2-propanol	10 s	[76]
PAHs	Reference marine sediment Reference soil	Dichloromethane Dichloromethane-toluene (50:50)	10 min, 30 W, open vessel	[77]

TABLE 3.4 (*continued*)

Analyte	Matrix	Solvent	MAE conditions	Reference
	Reference liver sediment	Acetone hexane (50:50,		
	Reference sewage sludge	60:40)		
	Industrial soil	Acetone		
	Marine sediment			
C_{60}	Fullerene soot	Toluene–acetonitrile (95:5)	20 min at 130°C,	[78]
C_{70}			closed vessel	
PCBs	Municipal sewage sludge	Hexane–acetone (1:1)	10 min, 30 W, open vessel	[79]
Organochlorine pesticides	Fish tissue	Ethylacetate–cyclohexane	Open vessel	[80]
PCBs	Blubber		Closed vessel	
Irganox 1010	Polypropylene	2-Propanol	150°C 140°C	[81]
		Acetone		
		Chloroform		
		Cyclohexane-2-propanol		
PCBs	River sediments	Acetone–hexane (1:1)	15 min, closed vessel	[82]
Atrazine	Sandy loam	Methanol		
Simazine	Clay	Acetone-hexane (1:1)		
Prometryne	Bentonite	Dichloromethane		[83]
	Florisil	Dichloromethane		
		Water		
Edible yellow pigment	Cape jasmine			[84]
Organochlorine pesticides	Penguin eggs	Etylacetate–cyclohexane		[85]
	Skua eggs			

Dimethomorph	Silt loam Clay loam Sandy loam	90% Acetonitrile–10% water 50% Methanol–50% water	3 min at 125°C, closed vessel	[88]
Seven organochlorine pesticides	Sediments	Acetone hexane–acetone (1:1) Tetrahydrofuran	5, 10, 20, and 30 min at 100 and 120°C, closed vessel	[89]
Atrazine	Soil	Water	3, 4, and 5 min, closed vessel	[90]
Phenol 2-Chlorophenol 2-Methylphenol 2-Nitrophenol 2,4-Dichlorophenol	Soil	Acetone–hexane (various ratios)	Closed vessel	[91]
Amino acids	Foods			[92]
Sulfamethazine	Swine tissue (muscle, liver, kidney)	Methanol	25 s	[93]
Hexaconazole	Sandy loam Sandy clay	Acetone	15 min at 115°C	[94]
PAHs	Soil	Acetone Dichloromethane	20 min at 120°C, closed vessel 20 min 300 W, open vessel	[95]
Taxanes, paclitaxel, 10-deacetyltaxol, cephalomannine	Taxus biomass (stems, needles)	Methanol	Closed vessel, 70, 85 and 115°C, 6, 9.1 and 12.2 min	[96]

TABLE 3.4 (*continued*)

Analyte	Matrix	Solvent	MAE conditions	Reference
L-hydroxy proline	Meat	50% HCl solution	15 min at 800 W, closed vessel 2 h at 200 W, open vessel	[98]
Organotin compounds	Sediments	11 M acetic acid NaBEt$_4$	3 min at 50–60 W, open vessel	[99]
Organomercury compounds	Sediments	2 M nitric acid 2 M hydrochloric acid	3 min at 60 W, open vessel	[99]
	Reference biological materials	25% tetramethyl-ammonium hydroxide	2–4 min at 40-60 W, open vessel	

was added to the soil matrix to bring its water content to 30%). Since water has a high dielectric constant, the temperature inside the vessel raises very rapidly when water is present. This sudden raise in temperature probably facilitates partitioning of the more volatile compounds into the headspace. Therefore, recoveries of the more volatile PAHs such as acenaphthene and acenaphthylene decreased by about 15–20% and of naphthalene by as much as 50%.

Other successful microwave-assisted extractions of PAHs from soils, sediments, and fly ash have been reported with hexane–acetone (1:1) [8,11,24,30,34,37,39,53,77], acetone alone [22,43,50,95], dichloromethane alone [22–24,35,45,50,53,77,95], dichloro-methane–toluene (50:50) [24,77], acetone–petroleum ether (1:1) [53], methanol–toluene (9:1) [53], and toluene–water [74].

Dean et al. [22] reported on a direct comparison between Soxhlet, MAE, and SFE for PAHs and concluded that the major advantage of MAE is the speed of extraction, but they also acknowledged that without additional cooling after extraction it takes approximately 30 min until the vessels will be opened and extracts processed. Barnabas et al. [43] also investigated effects of pressure, temperature, extraction time, and percent of methanol modifier added to the extraction solvent in order to optimize the extraction. During initial evaluation studies, these authors reported arcing when 5 g of soil sample were extracted with 30 ml solvent. Thus, they reduced the sample size to 2 g and increased the volume of solvent to 40 ml. From the experiments conducted (e.g. temperature: 40–120°C; extraction time 5–20 min; solvent volume 30 and 46 ml), Barnabas et al. concluded that MAE recoveries and Soxhlet recoveries were comparable, but method precision (%RSD) was far better for MAE than Soxhlet extraction [43].

Chee et al. [53] reported a 5-min heating at 115°C with 30 ml hexane–acetone (1:1) as the optimum extraction conditions for a 5-g sample, conditions which are very similar to those employed by Lopez-Avila et al. [7,8].

Optimization of MAE of PAHs using open-vessel technology was conducted by Budzinski et al. [77], who reported that the optimum conditions are 30% water, 30 ml dichloromethane, and 10-min heating at 30 W power. When considering that the time needed to reach the boiling point is about 2 min (for dichloromethane), a heating time of 10 min is more than sufficient to extract PAHs quantitatively from the matrix especially when adding water which is supposed to cause swelling of the matrix.

Organochlorine pesticides (OCPs): MAE of OCPs was reported by Onuska and Terry [5], Lopez-Avila et al. [7–9,70], Fish and Revesz [29], Li et al. [30], Chee et al. [50,54], Hummert et al. [55], McMillin et al. [60], Vetter et al. [80,87], Pastor et al. [74], Weich-brodt et al. [85], and Silgoner et al. [89].

Onuska and Terry [5] extracted aldrin, dieldrin, and DDT from soils and sediments using acetonitrile, isooctane or a mixture of isooctane–acetonitrile (1:1) and achieved quantitative recoveries using five or seven 30-s irradiations with microwave energy. They also reported that MAE recoveries increase as the moisture content of the soil increases up to 15%. Fish and Revesz [29] used hexane–acetone as extraction solvent and reported that OCP recoveries improved when changing from 1:1 hexane–acetone to 2:3 hexane–acetone. The latter solvent has a composition similar to the azeotropic vapor in the Soxhlet extractor.

Lopez-Avila et al. [9] extracted 45 OCPs from freshly spiked and 24-h aged soil samples with hexane–acetone (1:1). Table 3.5 shows the MAE recoveries data for a freshly

TABLE 3.5

MAE METHOD PERFORMANCE FOR THE COMPOUNDS LISTED IN METHOD 8081[a]

Compound no.	Compound name	Spike level (mg/kg)	Freshly spiked topsoil		Spiked topsoil aged for 24 h at 4°C		% Blowdown evaporation recovery
			% Average recovery[a]	% RSD	% Average recovery[a]	% RSD	
1	Alachlor	200	87.6	2.3	61.9	4.7	121
2	Aldrin	20	87.0	2.1	90.5	5.0	89.9
3	α-BHC	20	94.4	4.1	89.8	7.6	90.9
4	β-BHC	20	90.4	3.6	107	10	102
5	γ-BHC	20	89.6	1.3	93.6	7.0	106
6	δ-BHC	20	96.9	2.8	92.5	7.1	92.8
7	Captafol	200	122	4.7	36.1	6.9	105
8	Captan	200	105	1.7	20.9	30	110
9	Chlorobenzilate	100	82.6	5.6	97.9	5.4	98.3
10	α-Chlordane	20	80.0	3.9	83.2	3.5	99.2
11	γ-Chlordane	20	86.2	2.9	86.9	2.5	94.0
12	Chloroneb	100	69.6	2.4	126	18	87.5
13	Chloropropylate	100	86.0	5.6	102	5.4	94.4
14	Chlorothalonil	60	83.4	0.9	62.6	3.4	119
15	Dibromochloropropane	100	55.1	20	84.0	9.0	73.3
16	Dacthal	60	93.3	1.5	92.0	7.1	100
17	4,4'-DDD	20	76.9	4.5	73.2	7.6	115
18	4,4'-DDE	20	84.7	3.1	73.3	4.1	112
19	4,4'-DDT	20	116	5.6	123	10	93.3
20	Diallate	200	98.6	4.1	93.8	7.6	87.0
21	Dichlone	200	77.7	2.6	9.7	56	116
22	Dichloran	60	110	9.3	109	13	78.9
23	Dieldrin	20	85.9	3.8	90.4	3.9	95.8

24	Endosulfan I	20	86.8	3.1	85.8	4.6	97.9
25	Endosulfan II	20	71.9	6.3	66.5	2.9	112
26	Endosulfan sulfate	20	81.6	6.5	72.4	5.5	105
27	Endrin	20	97.4	1.9	103	11	102
28	Endrin aldehyde	20	78.3	3.5	58.3	5.9	88.7
29	Endrin ketone	20	90.6	3.0	74.0	4.3	99.3
30	Etridiazole	100	92.6	14	90.9	16	96.7
31	Heptachlor	20	110	1.4	95.6	7.1	82.0
32	Heptachlor epoxide	20	95.3	2.7	81.1	5.0	97.0
33	Hexachlorobenzene	200	80.8	1.6	95.7	4.6	86.1
34	Hexachlorocyclopentadiene	100	107	12	111	7.9	66.0
35	Isodrin	20	103	1.3	102	3.3	78.1
36	Methoxychlor	60	90.9	2.7	81.8	5.1	110
37	Mirex	20	93.5	3.4	86.8	3.6	89.7
38	Nitrofen	100	90.2	3.6	84.9	6.3	112
39	Pentachloronitrobenzene (PCNB)	100	94.5	1.8	100	7.1	88.9
40	Perthane	200	86.8	4.0	79.2	4.8	94.2
41	Propachlor	100	91.5	8.4	63.0	4.5	118
42	trans-Nonachlor	20	81.7	5.4	81.2	4.0	99.8
43	cis-Permethrin	60	81.2	4.8	68.8	2.6	113
44	trans-Permethrin	60	103	32	80.3	7.1	116
45	Trifluralin	40	101	2.4	98.7	5.4	83.9

[a] Sample size was 5 g; solvent (hexane–acetone, 1:1) volume was 30 ml; extraction time 10 min; extraction temperature 115°C; power was set at 100% (nominally 1000 W). The number of determinations was three. The topsoil (pH 7.5; cation exchange capacity 14.6 mequiv./100 g; organic carbon 0.1%; water content 2.6%; sand 57.6%; silt 21.8%; and clay 20.6%) was obtained from Sandoz Crop Protection (Gilroy, CA). The extracts were analyzed by GC-ECD. The recoveries were corrected for losses during blowdown evaporation [39].

spiked topsoil, and the same topsoil sample spiked with the target analytes and aged for 24 h at 4°C (the water content of the aged soil was adjusted to 30% to make it more representative of real world samples). For the freshly spiked soil, 38 compounds had recoveries between 80 and 120%, six compounds had recoveries between 50 and 80%, and the recovery of captafol was above 120%. For the spiked soil samples aged for 24 h, 28 compounds had recoveries between 80 and 120%, 12 compounds had recoveries between 50 and 80%, three compounds including captafol, captan, and dichlone were poorly recovered, and chloroneb and 4,4'-DDT had recoveries above 120%.

When recoveries from freshly spiked soil were compared to those from spiked soil aged, it was found that the recovery of captafol dropped from 122 to 36%, the recovery of captan dropped from 106 to 21%, and the recovery of dichlone dropped from 78 to 10%. Captafol and captan appear to be quite stable upon irradiation of soil/solvent suspensions but dichlone was found to disappear upon irradiation of the solvent (the recovery of dichlone from solvent was only 5.5% after heating at 145°C for 5 min and 2.6% after 20 min at the same temperature). Microbial degradation may be responsible for the low recoveries of captafol and captan, whereas in the case of dichlone it is quite likely that this compound is not stable under the conditions used. Nonetheless, these recoveries are higher than those obtained by Soxhlet or sonication extraction.

Water samples can also be extracted by MAE; however, they have to be preconcentrated first on a membrane disk or some adsorbent material. Chee et al. [50] used C_{18}-membrane disks and then extracted the disks with 20 ml solvent (acetone and dichloromethane) in a closed-vessel MAE system at 80, 100 and 120°C for 1, 3, 5, and 10 min. Acetone was found to give higher recoveries than dichloromethane [50]. This approach would allow extremely low detection limits since several disks generated by processing a large volume of sample can be extracted in one vessel.

Vetter et al. [55,80,87] extracted OCPs from fatty tissues (e.g. seal blubber) with solvents such as hexane [55] and ethyl acetate (1:1) [80,87]. To transfer heat to hexane which is microwave transparent, disks of Weflon (2.5 cm in diameter × 0.3 cm thickness) were used in the extraction vessel. The yield of extractable fat and recoveries of OCPs after seven irradiation cycles were comparable to those obtained by Soxhlet extraction. Since ethyl acetate–cyclohexane (1:1) seems to extract more fat than *n*-hexane, a gel permeation chromatography step after extraction is a must.

PCBs: MAE of PCBs was reported by Lopez-Avila et al. [10], Onuska and Terri [42], Chee et al. [50], Pastor et al. [74], Dupont et al. [79], and Kodba and Marsel [82]. Lopez-Avila et al. used hexane–acetone (1:1) and reported that the average recoveries from typical soil matrices were greater than 70% for the Aroclors 1016 and 1260 and the method precision was better than 7%. Furthermore, there was no degradation of PCBs upon heating of solvent/soil suspensions with microwave energy. Three reference materials and 24 soils from a Superfund site, most of which contained Aroclors, were extracted by MAE and analyzed by both GC/ECD and enzyme-linked immunosorbent assay (ELISA). As ELISA is very sensitive and its detection range is quite narrow, the hexane–acetone extracts were first diluted with methanol and subsequently with the assay buffer (which contained 50% methanol) to bring the Aroclor concentrations to less than 5 ng/ml. These data [10] indicate excellent agreement between the certified Soxhlet/GC/ECD data and the MAE-ELISA data (correlation coefficient 0.9986; slope 1.0168) and the MAE-GC/ECD data and the MAE-ELISA data (correlation coefficient

0.9793; slope 1.0468). Aroclors 1248, 1260, and 1262 gave ELISA responses that agreed well with the ELISA response for Aroclor 1254, but the ELISA responses for Aroclors 1016, 1232, 1242, and 1268 were off by more than a factor of 2 (almost 3 in the case of Aroclor 1268), and for Aroclor 1221 results were off by a factor of 15. The reader should be aware that when using this technique, the ELISA results must be verified by GC or other means.

Other solvents used to successfully extract PCBs from environmental samples include isooctane [42], acetone and dichloromethane [50], and toluene–water [74].

Phenols: MAE of phenolic compounds was reported by Lopez-Avila et al. [7–9,11,39], Llompart et al. [32,64], Chee et al. [52], and Egizabal et al. [91]. Acetone–hexane seems to be the preferred solvent for 16 phenolic compounds regulated by the EPA and dichloromethane [52] and acetone–petroleum ether (1:1) [52] were reported to work well for extraction of nonyl phenol [52]. The only compounds found to degrade during MAE are 2,4-dinitrophenol and 4,6-dinitro-2-methylphenol [7,10]. All reports in the literature indicate that MAE recoveries for phenolic compounds are higher than the classical extraction method recoveries and the method precision is significantly better for MAE (e.g. coefficient of variation of 3% for MAE as compared to 15% for Soxhlet and 20% for sonication) [1].

Herbicides: Immidazolinones (e.g. imazapyr, imazmetapyr, imazethapyr, imazaquin, etc.), a relatively new class of herbicides from American Cyanamid Company with a low-use rate, are extracted from soil with 0.1 M ammonium acetate/ammonium hydroxide (pH 9–10) in a 10-min extraction [25,36,38]. A variety of soil samples fortified at 1–50 ppb exhibited an average recovery of 92% (standard deviation 13%).

Triazine herbicides have been successfully extracted from soil by MAE with water [14,83,90], methanol [83], acetone–hexane (1:1) [83], dichloromethane [83], acetonitrile–0.5% ammonia in water (70:30) [63], dichloromethane–water (50:50) [63], methanol–dichloromethane (10:90) [63]. Water seems to be preferred since it is a very polar solvent and can interact strongly with polar matter in soils to enhance the desorption of triazines [83], it is a cheap, safe and environmentally friendly solvent, and it heats up very quickly when irradiated with microwave energy. Xiong et al. [90] reported that direct heating of soil with water gave a 73.4% recovery for atrazine from soil and therefore stated that ''MAE is not only a simple heating''. Using water as the extraction solvent, Xiong et al. [90] reported on the use of MAE coupled with ELISA to analyze atrazine in soil at concentrations of 5 ppb.

Organotin and organomercury compounds: Methods reported in the literature for the determination of organotin compounds in soils use extraction with organic solvents in the presence of a complexing agent or leaching with acetic or hydrochloric acid assisted by sonication or some sort of shaking. Open-vessel MAE was recommended to accelerate the leaching with 50% acetic acid aqueous solution and the data showed that a 3-min irradiation at 60 W was sufficient to recover tributyl tin from certified reference sediments [18]. Ethanoic acid (0.5 M in methanol) was also reported [46]. When dealing with biological matrices (e.g. tuna tissue, mussel tissue) solubilization with tetramethyl ammonium hydroxide (TMAH) for 3 min at 90, 115 and 130°C in a closed vessel was demonstrated to be as efficient as the hot-plate procedure [72]. Schmitt et al. [99] reported on the integration of the solubilization step with the derivatization/extraction step by using 11 M acetic acid and NaBEt$_4$.

Organomercury compounds can be extracted from sediments with 6 M hydrochloric acid at 120°C for 10 min in a closed vessel [67] or 2 M nitric acid and 2 M hydrochloric acid after 3 min irradiation at 60 W in an open vessel [99]. Pure acetic acid and 1 M sulfuric acid could only extract 85 and 55%, respectively. Microwave-assisted digestion of the biological tissue with 25% TMAH for 2–4 min at 40–60 W gave quantitative recovery of both organomercury and inorganic mercury [99].

3.4.2 Other applications

Additives in polymers:. Antioxidants such as Irganox 1010, Irganox 1076 and Irgaphos 168 are added to polymers to protect them during processing and end-use applications. Analytical methods for the determination of these compounds involve refluxing the polymer with a solvent for 8–48 h followed by chromatographic analysis. Studies involving MAE [6,28,81] indicate that such compounds are extracted with >95% efficiency with *n*-heptane-acetone in 6 min [6].

While some polymers are easily dissolved at room temperature, others such as polyethylene require elevated temperatures to dissolve them completely. Jordi et al. [28] used temperatures in excess of 140°C and cyclohexane–chloroform–triethylamine (45:45:10) to dissolve polyethylene and extract hindered amine light stabilizers (HALS) such as Tinuvin 770, Tinuvin 622, Tinuvin 144, and Chimassorb 81 (cyclohexane was the polymer swelling solvent and chloroform and triethylamine were added to enhance the extraction yields of the HALS. Additional comments on selecting solvents for MAE of polymers are given in Section 3.2.2.

Natural products: In 1986 Ganzler's first report on the use of MAE was on the extraction of vicine and convicine from fava beans [3], and gossypol from cotton seeds [3,4]. Lupin alkaloids (e.g. spateine) were extracted by Ganzler et al. with methanol–acetic acid and methanol ammonia with about 20% greater recovery than by conventional Soxhlet extraction [15].

Extraction of oils from mint leaves and other materials of biological origin is a patented process [101] known as the microwave-assisted process or MAP. Other reports on MAE of natural products include that of Young [21], Bichi et al. [26], and Mattina et al.[96]. Young [21] reported on the extraction of ergosterol from fungi and spores, mushrooms, filtered air, contaminated corn and grain dust. The samples were suspended in methanol (2 ml) and treated with 2 M aqueous sodium hydroxide and irradiated with microwave energy for 35 s (375 W power). Bichi et al. [26] extracted pyrrolizidine alkaloids from *Senecio palvadosos* and *Senecio cordatus* dried plants by MAE with methanol at 65–100°C for 20–30 min. and reported that the MAE extracts were identical to those obtained by Soxhlet extraction with methanol. Mattina et al. [96] demonstrated that it is possible to extract taxanes from Taxus biomass by MAE with 1/10 the volume of methanol used in the conventional extraction and also reported that use of 95% ethanol would significantly reduce the costs of the extraction without reduction in taxane recoveries. Using 5 g of freshly harvested needles (moisture content 55–65%) soaked in 5 ml water prior to MAE and 10 ml ethanol at 85°C for 9 min about 90% of the taxanes present in the sample are recovered by MAE.

3.4.3 Comparison of MAE with Soxhlet extraction, sonication, and SFE

The extractability of 95 compounds listed in EPA Method 8250 using MAE, Soxhlet,

sonication, or SFE was investigated by Lopez-Avila et al. [11]. Freshly spiked soil samples and two standard reference materials were extracted with hexane–acetone (1:1) for MAE and Soxhlet extraction, with methylene chloride–acetone (1:1) for sonication extraction, and with supercritical carbon dioxide modified with 10% methanol for SFE. Whereas the MAE results look very encouraging when compared with the results obtained by the other extraction techniques, the MAE of a group of 15 polar basic compounds at 115°C with hexane–acetone for 10 min (1000 W power) gives poor recoveries. Since none of the other techniques seem to provide acceptable recoveries for these polar compounds, we investigated the MAE with acetonitrile at 50 and 115°C and present the results of these experiments.

Table 3.6 summarizes the results from the evaluation of the four extraction techniques. Of the 94 average recovery values presented in Table 3.6 under the MAE technique, 51 values were above 80%, 33 ranged from 50 to 79%, eight from 20 to 49%, and two were below 19%. The distribution of the Soxhlet data was very similar to that of MAE data (50 recoveries were above 80%, 32 ranged from 50 to 79%, eight from 20 to 49%, and four were below 19%). In contrast, the sonication recoveries appeared to be slightly higher than either the MAE or Soxhlet recoveries (63 values were above 80%, 25 ranged from 50 to 79%, four from 20 to 49%, and two were below 19%). The SFE recoveries were the lowest among the four techniques (37 values were above 80%, 37 values ranged form 50 to 79%, 12 from 20 to 49%, and eight values were below 19%).

The MAE technique gave the best precision (percent RSDs were equal to or less than 10% for 90 of the 94 RSD values in Table 3.6, MAE column). The Soxhlet technique gave the worst precision or the highest percent RSDs (only 52 of the 94% RSD values were equal to or less than 10%).

Table 3.7 summarizes the percent average recoveries and percent RSDs for 23 compounds known to be present in the ERA soil. The certified levels given in Table 3.7 are actually the ''true spike'' concentrations since this material is a soil spiked with the target compounds and weathered. The QC acceptance criteria established by EPA for Method 8250 are also included in this table. When comparing the data obtained for the unspiked ERA soil by the four extraction techniques, it was concluded that for most of the target compounds, MAE performed better than the Soxhlet or the sonication extraction (the ratios of recoveries obtained by MAE and Soxhlet extraction ranged from 1 to 2.16 except for benzo(b + k)fluoranthene at 0.82; the ratios of recoveries obtained by MAE and sonication extraction ranged from 1 to 2.16 except 0.7 for bis(2-ethylhexyl) phthalate, 0.86 for butyl benzyl phthalate, and 0.92–0.98 for three compounds. With the exception of the two dichlorobenzenes and 1,2,4-trichlorobenzene, all the other recovery data in Table 3.7 are within the QC acceptance criteria established by EPA. The low recoveries of the two dichlorobenzenes and of 1,2,4-trichlorobenzene achieved by the four methods may not necessarily be due to inefficient extraction from the matrix, but to losses during extract manipulation after extraction (e.g. concentration from an initial volume of 300 ml to a final volume of 1 ml for Soxhlet and sonication extraction) or to inefficient collection during SFE. Another explanation would be that the certified values are not correct. The latter explanation is more plausible in this case since results [11] indicated that recoveries of chlorinated benzenes were greater than 80% when freshly spiked soil samples were extracted by MAE and only slightly higher than 30% for soil samples aged for 24 h.

The experiments performed with acetonitrile (Table 3.8) at 50 and 115°C indicate that

TABLE 3.6

PERFORMANCE OF MAE, SOXHLET, SONICATION, AND SFE FOR METHOD 8250 COMPOUNDS FROM SPIKED ERA SOIL LOT 330[a]

Compound no.	Compound name	Spike level (mg/kg)	MAE with hexane–acetone (1:1)[b]		Soxhlet extraction with hexane–acetone (1:1)[b]		Sonication extraction methylene chloride-acetone (1:1)[b]		SFE with carbon dioxide with 10% methanol[b]	
			% average recovery	% RSD	% average recovery	% RSD	% average recovery	% RSD	% average recovery	% RSD
1	Acenaphthene	5.0	93.5	1.3	88.1	11	93.0	5.5	69.4	5.5
2	Acenaphthylene	5.0	92.9	1.7	70.6	8.0	90.9	5.0	74.0	6.2
3	Acetophenone	5.0	66.9	5.3	58.6	7.0	70.3	7.6	50.9	8.8
4	4-Aminobiphenyl	5.0	75.9	12	44.9	7.1	91.6	5.6	38.2	29
5	Aniline	5.0	55.3	2.0	32.6	22	67.2	14	61.7	20
6	Anthracene	5.0	79.4	0.7	68.7	14	74.4	9.2	70.7	4.1
7	Benzidine	5.0	4.6	27	ND[c]		38.2	33	ND	
8	Benzoic acid	5.0	42.6	1.4	163	24	56.1	35	102	13
9	Benzo(a)anthracene	5.0	104	1.2	94.5	16	104	6.4	89.9	0.8
10	Benzo(b)fluoranthene ⎱	5.0	104	0.9	109	11	95.9	6.8	87.8	6.3
11	Benzo(k)fluoranthene ⎰	5.0								
12	Benzo(g,h,i)perylene	5.0	113	2.3	92.7	2.9	117	6.7	88.6	1.8
13	Benzo(a)pyrene	5.0	98.4	1.4	86.4	16	85.0	8.7	77.0	4.7
14	Benzyl alcohol	5.0	78.5	3.5	78.3	6.7	79.8	4.6	72.3	9.1
15	Bis(2-chloroethoxy) methane	5.0	73.5	3.6	72.8	9.0	79.7	5.7	57.4	7.9
16	Bis(2-chloroethyl) ether	5.0	63.0	6.2	62.9	7.0	61.8	12	73.1	27
17	Bis(2-chloroisopropyl) ether	5.0	62.4	5.3	62.1	8.2	60.8	8.3	42.5	14
18	Bis(2-ethylhexyl) phthalate	5.0	107	1.5	110	9.7	112	3.2	87.8	5.8
19	4-Bromophenyl phenyl ether	5.0	107	1.0	97.7	14	100	6.2	86.8	1.3
20	Butyl benzyl phthalate	5.0	103	0.1	105	12	103	4.5	81.3	4.6
21	4-Chloroaniline	5.0	61.5	9.3	27.2	24	82.6	16	61.0	13
22	1-Chloronaphthalene	5.0	88.1	2.2	82.4	8.0	83.8	4.7	54.7	9.2
23	2-Chloronaphthalene	5.0	87.0	2.5	82.3	11	88.6	4.6	67.0	5.4
24	4-Chloro-3-methylphenol	5.0	94.1	4.7	97.2	16	90.5	7.9	89.7	7.6
25	2-Chlorophenol	5.0	69.1	4.1	68.0	6.8	71.5	8.3	54.3	12

26	4-Chlorophenyl phenyl ether	5.0	97.5	1.5	100	13	95.2	5.7	80.5	4.2
27	Chrysene	5.0	111	2.3	106	16	108	6.5	95.0	3.8
28	Dibenzo(a,j)acridine	5.0	26.4	14	103	7.6	104	7.2	10.2	80
29	Dibenzo(a,h)anthracene	5.0	117	1.3	102	5.8	114	5.9	99.5	3.3
30	Dibenzofuran	5.0	99.4	2.1	93.6	12	90.9	5.5	75.3	5.6
31	Di-n-butyl phthalate	5.0	110	0.1	98.9	13	106	7.3	79.0	1.7
32	1,2-Dichlorobenzene	5.0	36.0	4.4	33.1	17	30.0	10	17.2	19
33	1,3-Dichlorobenzene	5.0	56.7	4.1	52.3	14	49.6	10	28.7	32
34	1,4-Dichlorobenzene	5.0	36.0	4.7	33.2	16	30.7	11	14.5	15
35	3,3'-Dichlorobenzidine	5.0	96.5	3.8	46.5	13	109	14	77.7	16
36	2,4-Dichlorophenol	5.0	84.6	2.9	89.1	18	83.5	5.5	76.3	13
37	2,6-Dichlorophenol	5.0	86.3	2.1	80.6	12	93.1	5.2	72.9	11
38	Diethyl phthalate	5.0	111	1.7	103	15	98.1	5.7	86.9	2.5
39	Dimethylaminoazobenzene	5.0	115	0.6	98.8	13	113	7.7	94.2	2.8
40	7,12-Dimethylbenz(a)anthracene	5.0	103	3.4	120	17	90.6	12	98.7	3.2
41	α,α-Dimethylphenethylamine	5.0	ND		ND		ND		ND	
42	2,4-Dimethylphenol	5.0	73.8	6.3	54.9	24	89.0	6.3	65.5	17
43	Dimethyl phthalate	5.0	105	0.8	96.2	13	101	5.6	82.2	2.9
44	4,6-Dinitro-2-methylphenol	5.0	70.2	4.1	87.3	6.9	153	6.4	81.4	8.2
45	2,4-Dinitrophenol	5.0	51.7	1.6	85.5	1.1	107	15	80.3	1.4
46	2,4-Dinitrotoluene	5.0	112	0.4	107	19	92.7	8.2	104	8.1
47	2,6-Dinitrotoluene	5.0	104	0.3	105	15	94.7	7.7	94.3	6.4
48	1,2-Diphenylhydrazine[d]	5.0	113	1.1	104	16	116	5.5	93.5	2.5
49	Di-n-octyl phthalate	5.0	124	2.8	137	20	99.8	7.6	81.6	5.1
50	Ethyl methanesulfonate	5.0	62.1	4.8	65.3	8.2	69.2	7.6	52.8	5.8
51	Fluoranthene	5.0	104	1.4	91.5	12	96.1	6.6	80.0	2.4
52	Fluorene	5.0	103	1.3	93.4	14	94.5	6.2	78.8	5.0
53	Hexachlorobenzene	5.0	107	0.7	97.7	13	98.6	5.9	86.5	1.3
54	Hexachlorobutadiene	5.0	64.2	5.2	60.1	10	61.1	11	38.8	13
55	Hexachlorocyclopentadiene	5.0	26.7	12	15.5	73	ND		ND	
56	Hexachloroethane	5.0	59.1	7.2	53.3	24	52.2	20	28.2	21
57	Indeno(1,2,3-cd)pyrene	5.0	114	1.6	96.4	7.4	125	6.9	97.0	4.1
58	Isophorone	5.0	75.9	2.9	74.3	9.8	82.7	4.8	66.8	5.0
59	3-Methylcholanthrene	5.0	118	0.6	84.0	1.8	151	10	151	3.1
60	Methyl methanesulfonate	5.0	44.4	7.2	62.3	11	61.0	8.9	40.4	24

TABLE 3.6 (*continued*)

Compound no.	Compound name	Spike level (mg/kg)	MAE with hexane–acetone (1:1)[b]		Soxhlet extraction with hexane–acetone (1:1)[b]		Sonication extraction methylene chloride-acetone (1:1)[b]		SFE with carbon dioxide with 10% methanol[b]	
			% average recovery	% RSD	% average recovery	% RSD	% average recovery	% RSD	% average recovery	% RSD
61	2-Methylnaphthalene	5.0	77.5	3.7	74.2	8.0	86.0	4.8	53.9	15
62	2-Methylphenol	5.0	57.7	4.1	58.8	11	63.3	8.3	48.2	8.5
63	4-Methylphenol	5.0	66.7	4.1	66.8	8.8	68.5	7.5	57.2	9.9
64	Naphthalene	5.0	63.3	5.0	60.4	7.3	62.5	7.7	41.7	8.9
65	1-Naphthylamine	5.0	32.0	6.8	25.4	19	75.0	11	15.3	47
66	2-Naphthylamine	5.0	56.2	9.9	14.9	41	72.9	5.6	4.9	43
67	2-Nitroaniline	5.0	109	0.8	99.5	15	102	7.0	85.5	3.5
68	3-Nitroaniline	5.0	100	1.8	74.3	17	108	8.1	76.7	9.2
69	4-Nitroaniline	5.0	105	1.5	84.4	16	110	10	86.0	4.1
70	Nitrobenzene	5.0	67.3	5.0	65.3	8.6	69.2	7.3	42.0	27
71	2-Nitrophenol	5.0	67.8	4.7	67.4	8.2	81.5	14	57.5	5.1
72	4-Nitrophenol	5.0	95.6	3.6	97.1	14	107	12	99.6	4.5
73	N-nitroso-di-n-butylamine	5.0	88.4	1.3	87.6	12	94.3	7.3	78.1	4.6
74	N-nitroso-di-n-propylamine	5.0	65.0	5.6	71.7	4.0	75.8	3.6	56.5	16
75	N-nitrosopiperidine	5.0	74.1	3.4	74.2	7.8	77.3	5.8	62.8	16
76	Pentachlorobenzene	5.0	100	0.8	94.3	14	94.3	4.3	74.3	7.3
77	Pentachloronitrobenzene	5.0	117	1.0	107	15	121	8.2	103	2.0
78	Pentachlorophenol	5.0	58.0	3.7	77.1	9.3	72.1	17	56.3	8.3
79	Phenacetin	5.0	107	2.5	104	14	103	7.8	101	1.3
80	Phenanthrene	5.0	107	0.7	97.6	14	98.5	7.9	86.0	2.7
81	Phenol	5.0	75.2	3.0	76.5	8.9	81.2	1.1	88.6	14
82	2-Picoline	5.0	19.7	8.5	47.6	13	38.1	17	31.1	35
83	Pronamid	5.0	110	1.4	96.9	14	105	2.9	96.7	3.3
84	Pyrene	5.0	96.6	0.6	98.2	9.1	93.4	6.3	72.6	4.7
85	1,2,4,5-Tetrachlorobenzene	5.0	82.6	3.1	76.7	8.8	83.2	5.2	56.1	8.2
86	2,3,4,6-Tetrachlorophenol	5.0	110	1.2	103	16	118	12	103	3.8

87	1,2,4-Trichlorobenzene	5.0	58.7	5.2	55.2	9.3	58.2	11	36.3	9.8
88	2,4,5-Trichlorophenol	5.0	115	0.8	101	13	92.3	5.6	88.2	4.2
89	2,4,6-Trichlorophenol	5.0	96.4	1.2	98.3	14	93.1	8.3	91.7	0.9
SA-1	2-Fluorobiphenyl	2.5	84.1	3.7	78.8	11	84.5	4.8	63.4	5.1
SA-2	2-Fluorophenol	5.0	58.4	4.2	56.3	7.1	63.7	9.3	50.6	11
SA-3	Nitrobenzene-d_5	2.5	63.0	5.1	60.0	7.8	64.6	7.3	47.0	12
SA-4	Phenol-d_5	5.0	70.4	3.9	69.9	7.6	77.6	5.4	62.7	12
SA-5	Terphenyl-d_{14}	2.5	102	1.0	97.7	15	94.5	7.4	69.6	4.0
SA-6	2,4,6-Tribromophenol	5.0	110	1.0	98.6	15	98.2	9.6	91.1	3.5

[a] This soil was obtained from Environmental Resource Associates (ERA) in Arvada, CO, and has been certified for the compounds listed in Table 3.2.

[b] The number of determinations was three. The recoveries are based on the ERA certified values plus the amounts spiked and were not corrected for losses incurred during blowdown evaporation.

[c] ND, not detected.

[d] The soil samples were spiked with azobenzene since 1,2-diphenylhydrazine decomposes to azobenzene during sample work-up and analysis.

Ref. [11], with permission.

TABLE 3.7

PERFORMANCE OF MAE, SOXHLET, SONICATION, AND SFE FOR SELECTED METHOD 8250 COMPOUNDS FROM ERA SOIL LOT 330[a]

Compound no.	Compound name	Certified spike level (mg/kg)[c]	MAE with hexane–acetone (1:1)[c]		Soxhlet extraction with hexane–acetone (1:1)[c]		Sonication extraction with methylen chloride-acetone (1:1)[c]		SFE with carbon dioxide with 10% methanol[c]		QC acceptance criteria established by EPA[b]
			% average recovery	% RSD	% average recovery	% RSD	% average recovery	% RSD	% average recovery	% RSD	
Native compounds											
6	Anthracene	7.25	69.0	4.4	46.9	12	46.5	22	55.6	7.5	27-133
10	Benzo(b)fluoranthene	6.47	101	5.8	122	13	66.7	13	74.5	7.4	24-159
11	Benzo(k)fluoranthene										11-162
13	Benzo(a)pyrene	2.92	67.1	6.2	57.0	4.6	39.4	6.5	53.0	12	17-163
18	Bis(2-ethylhexyl) phthalate	6.87	118	1.5	118	6.3	170	38	103	7.5	8-158
20	Butyl benzyl phthalate	3.88	113	1.0	113	6.0	131	26	90.4	6.5	D-152
24	4-Chloro-3-methylphenol	5.14	89.9	0.8	76.9	4.9	73.6	13	86.4	9.0	22-147
26	4-Chlorophenyl phenyl ether	4.84	96.9	2.8	95.6	7.5	83.4	15	70.0	7.5	25-158
27	Chrysene	2.67	113	1.9	107	6.7	79.8	14	86.7	6.0	17-168
30	Dibenzofuran	3.78	89.6	1.8	88.8	11	76.7	17	63.6	6.3	[d]
32	1,2-Dichlorobenzene	8.76	20.7	5.8	17.6	32	20.4	18	9.9	16	32-129
34	1,4-Dichlorobenzene	5.13	13.3	5.6	10.5	28	14.0	21	5.9	15	20-124
36	2,4-Dichlorophenol	9.05	69.8	1.1	51.7	15	64.3	26	65.6	9.2	39-135
46	2,4-Dinitrotoluene	6.93	98.6	2.6	80.3	16	67.5	39	72.4	10	39-139

47	2,6-Dinitrotoluene	5.22	95.4	2.3	88.1	8.1	73.7	33	72.1	11	50-158
62	2-Methylphenol	12.20	50.5	1.2	32.9	15	48.9	18	36.4	11	[d]
63	4-Methylphenol	8.70	53.9	0.4	42.6	5.5	55.7	21	40.7	15	[d]
64	Naphthalene	3.53	50.2	3.3	44.1	26	49.1	17	33.4	13	21-133
74	N-nitroso-di-n-propylamine	5.44	68.8	2.0	73.6	23	74.8	13	55.8	15	D-230
78	Pentachlorophenol	14.60	72.4	3.0	65.5	3.2	33.5	72	52.1	17	14-176
84	Pyrene	6.00	107	1.1	102	7.0	141	49	77.5	7.2	52-115
87	1,2,4-Trichlorobenzene	2.75	39.8	4.1	32.9	30	39.8	18	24.4	14	44-142
89	2,4,6-Trichlorophenol	6.49	89.5	2.0	41.3	47	67.4	27	82.5	4.5	37-144

Spiked compounds

SA-1	2-Fluorobiphenyl	5.0	77.7	1.2	74.6	14	78.9	10	52.9	14	30-115
SA-2	2-Fluorophenol	5.0	60.4	3.8	50.2	23	62.1	15	49.8	15	25-121
SA-3	Nitrobenzene-d_5	5.0	61.7	2.7	54.9	22	66.5	11	41.0	16	23-120
SA-4	Phenol-d_5	5.0	69.4	1.3	65.5	21	73.4	13	57.1	15	24-113
SA-5	Terphenyl-d_{14}	5.0	109	1.9	92.4	20	151	36	77.3	7.6	18-137
SA-6	2,4,6-Tribromophenol	5.0	102	1.0	51.8	50	64.1	43	80.0	1.3	19-122

[a] This soil was obtained from Environmental Resource Associates (ERA) in Arvada, CO, and has been certified for the compounds listed in this table.

[b] Reported by ERA.

[c] The number of determinations was three. The recoveries are based on the ERA certified values and were not corrected for losses incurred during blowdown evaporation.

[d] No value given.

Ref. [11], with permission.

TABLE 3.8

MAE RECOVERIES OF SELECTED BASIC COMPOUNDS FROM FRESHLY SPIKED SOIL USING ACETONITRILE[a]

Compound no.	Compound name	Blowdown evaporation	50°C (solvent only) % average recovery	50°C (spiked soil) % average recovery	% RSD	115°C (solvent only) % average recovery	115°C (spiked soil) % average recovery	% RSD
4	4-Aminobiphenyl	100	93.3	81.0	3.1	92.1	79.4	1.9
5	Aniline	98.9	75.8	57.0	13	75.7	63.0	7.7
7	Benzidine	ND[b]	–	–	–	–	–	–
21	4-Chloroaniline	101	102	79.8	7.6	94.2	85.4	1.5
28	Dibenzo(a,j)acridine	81.5	84.4	49.2	25	85.4	75.1	10
35	3,3'-Dichlorobenzidine	108	107	102	1.3	105	106	1.3
39	Dimethylaminoazobenzene	101	105	100	4.4	104	99.4	2.7
41	α,α-Dimethylphenethyl-amine	81.7	86.5	0	–	89.6	0	–
48	1,2-Diphenylhydrazine[c]	98.5	98.5	84.7	4.5	95.4	83.5	1.3
65	1-Naphthylamine	100	104	82.1	6.1	87.6	53.6	20
66	2-Naphthylamine	100	65.5	63.2	21	54.9	71.4	4.8
67	2-Nitroaniline	99.9	96.5	91.3	1.2	92.2	91.6	3.0
68	3-Nitroaniline	110	110	106	3.1	105	110	0.8
69	4-Nitroaniline	113	112	102	4.1	111	104	2.3
82	2-Picoline	34.5	31.7	17.0	26	28.2	19.5	4.9

[a] The spike level was 10 mg/kg. The number of determinations was two for "solvent only" experiments and three for "spiked soil" experiments.
[b] ND, not detected in the standard subjected to blowdown evaporation.
[c] Determined as azobenzene.
Ref. [11], with permission.

all polar compounds, except α,α-dimethylphenethylamine, could be recovered by MAE from the freshly spiked soil. It was found that at 115°C the average recoveries of 10 of the 14 compounds were greater than 70%. Benzidine was not recovered during the blowdown evaporation of the spiked acetonitrile and thus, it is not included in the group of 15 polar compounds. Two compounds (e.g. 1-naphthylamine and aniline) exhibited recoveries of 53.6 and 63%, respectively; and picoline exhibited a recovery of only 19.5% but its blowdown recovery was only 34.5%. Thus, a more polar solvent appears to be the answer for the efficient extraction of these type of compounds from the soil matrix.

3.5 FUTURE DIRECTIONS

Future instrument developments will likely focus on vessel design and materials, increased sample throughput by integrating extraction/centrifugation/solvent evaporation, and perhaps full integration of the microwave sample preparation with instrumental analysis. One step in this direction is a focused microwave-assisted Soxhlet reported recently by Luque de Castro et al. [102]. This device uses conventional Soxhlet glassware for sample extraction and a focused-microwave digester for irradiation of the sample cartridge at required intervals, while the fresh solvent continues to pass through the sample, and was shown to achieve reduction of the extraction times from 8 to 1 h. The future will see specialized fields developing beyond using microwave energy to heat up the solvent and or the matrix [1]. These will include: extraction for speciation, flow-through microwave extraction, in situ or on-line derivatization coupled with extraction. More information on future directions in microwave-assisted chemistry can be found in Ref. [1].

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Chapter 4

Sample handling and analysis of pesticides and their transformation products in water matrices by liquid chromatographic techniques

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CONTENTS

4.1 INTRODUCTION

After the recently published extensive compilation [1] on the analysis of pesticides and their transformation products in water, recent research in this area has taken different directions which will be discussed in this chapter.

One of the driving forces in the development of new analytical methods for pesticides is the fact that new metabolites are being found, and this is the main area where research still is needed. Every year new polar pesticide metabolites, like oxanilic acid derivatives from

alachlor, metolachlor and acetolachlor, or cyanazine acid and amide from cyanazine, are being added to recent monitoring programs, thus allowing us to extract new information from the environment and consequently develop better diagnostics. In this respect, the analysis of pesticide metabolites – all of which are included, in the European Union Directive for Drinking Water and should be at levels lower than 0.1 ppb – implies higher costs in the analysis of water samples. It is known that a method that is appropriate for a certain pesticide may not work for a more polar pesticide metabolite. In this context, for the current monitoring programs, at least two or more different analytical methods should be used sometimes for the correct analysis of certain pesticides and their metabolites. In the case of metabolites, it may also happen that a certain metabolite, detected for instance by tandem MS, is not commercially available and then synthesis is required for its unequivocal identification. Finally the problem of which metabolites to quantify and prioritize, as a function of toxicological importance, and to assign its importance as environmental indicators is also relevant.

The relevance of new metabolites that are being analyzed is closely linked to two steps in the determination of pesticides: sample handling and analysis. The development of improved and new sample handling protocols for the enrichment of very polar pesticides, which means difficult to analyze pesticides, e.g. glyfosate, and pesticide metabolites like those of the herbicide dimethyl tetrachloroterephthalate (DCPA) is currently taking place. Adsorbents like those of polymeric type such as Lichrolut EN and Isolute EN, carbon type or double layer cartridges are used now routinely for the trace enrichment of a wide variety of pesticides and their metabolites in water matrices in substitution of C18 sorbents. Among the new sample handling protocols we should mention the use of immunosorbents – involving specific antibodies usually covalently bound to a silica matrix – for an efficient cleanup of complex water samples and the recently developed molecular imprints. These two last types of solid phase extraction (SPE) materials are tailor-made sorbents and are being developed at research level and they are partly commercially available. In this chapter we will bring few examples of sample handling protocols for the enrichment of different groups of pesticides like chlorotriazine, carbamate, phenylurea and acidic pesticides.

The last driving force in the analysis of pesticides in water matrices is the increased development and application of LC-MS using mainly the different options of atmospheric pressure ionization interfaces, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The breakthrough point is that many MS manufacturers have developed in the last few years the so-called bench-top LC-MS instruments incorporating either API sources. Such devices with prices in the range of US$100–150 000 are very competitive and are being purchased by an increasing number of research and routine laboratories. Certainly, the advent of APCI and ESI-MS interfacing systems has expanded the applicability of LC-MS in environmental analysis mainly due to the high sensitivity and structural information obtained. Nowadays it is accepted that these two interfaces are the most applicable interfaces in the environmental area and can be considered in a way the standard interfaces for environmental analysis. The identification potential of LC-ESI-MS can be enhanced by using an ion trap, with several commercial manufacturers, or by using a MS-MS system. The combination of capillary electrophoresis (CE)-MS is also slowly growing, but in a minor degree as compared to LC-MS.

Certainly the combination of appropriate SPE protocols together with the sensitive LC-MS determinations has permitted an increase in the large number of metabolites or trans-

formation products that are being found or reported in the literature in the last few years. By the use of tailor-made sample handling techniques followed generally by LC-MS it is possible to identify unknown (today) pesticide metabolite. This is very important since these metabolites give information about the transformation process of the target pesticide in the environment. Chemical transformation – either hydrolysis and photolysis – and microbial metabolism result in alterations of the original molecule, subsequently altering its chemical property. With knowledge of the pesticide metabolites it is possible – with controlled experiments – to increase the knowledge of geochemical, microbial and physico-chemical processes, with a better understanding of the behavior of pesticides in specific environmental compartments that occur along the pesticide emission route [2].

From the top ten pesticides reported worldwide [1], atrazine is one of the most commonly used (and still analyzed) pesticides and many publications about atrazine appear each year. Most probably it is the pesticide with the highest number of publications –after DDT – mainly because it is very ubiquitous and easy to analyze. Metabolites of atrazine are increasingly being analyzed. For instance the contribution of hydroxylated atrazine degradation products to the total atrazine loads in Midwestern Streams of the US was recently assessed [3]. In this report it was concluded that atrazine metabolites accounted for nearly 60% of the atrazine load in northern Missouri streams at preplant, hydroxyatrazine being the predominant metabolite present. As regards the occurrence of pesticides in shallow groundwater samples of the US, it was also found that atrazine was the most frequently detected pesticide by 38.2% followed by deethylatrazine, 34.2%, whereas simazine, metolachlor and prometron were detected at percentages below 20% [4].

The problem of atrazine and related corn pesticides is mainly in the US and Europe, but in other countries, like Asian countries and Latin America, where rice is grown, other pesticides are of importance. One of the mostly used pesticides for rice cultivation is propanil, which is also one of the top ten pesticides in the world. In a recent paper [5] Propanil degradation was studied. This paper is a good example of the combination of advanced sample handling and analytical strategies applied to the trace determination of pesticides and their metabolites in natural waters. Propanil is usually sprayed before rice planting, over the dry fields, during the summer. Propanil it is a contact herbicide used world-wide to control weeds through inhibition of photosynthesis. A short duration of activity has been reported for propanil, with rapid degradation to 3,4-dichloroaniline (DCA), the major product formed. The main degradation pathway is through enzymatic hydrolysis (80–85%) with a lower photodegradation rate (6%). Propanil degradation is directly dependent on the environmental conditions and rate of application of the pesticide. In rice plants a fast degradation resulted in the total transformation of the pesticide within 5 days. The disappearance in water is usually correlated with absorption by rice foliage, but elevated soil salinity retarded its degradation. Table 4.1. reports the half-lives of propanil under different environmental and laboratory conditions. This indicates the importance of a good estimation of the half-lives under real and laboratory conditions and how the different experimental conditions can affect pesticide degradation. Propanil half-lives obtained at 35°C, under light irradiation, with and without humic acid addition were 2.95 and 1.73 days, respectively and are indicated in Table 4.1. These results suggest the strong influence of the organic matter over propanil transformation. It could be supposed that, at the real field pH (between 7.8 and 8.8), the organic matter (humics) should strongly

TABLE 4.1

COMPARATIVE DATA ON THE HALF-LIFE OF PROPANIL UNDER DIFFERENT CONDITIONS

Sample type and conditions (propanil concentration)	Half-life (days)	Ref.
Rice paddy water from Ebro Delta, 20–30°C, sunlight	1.24	[5]
Milli-Q spiked water (10 μg/l), 4°C, dark	12.27	[5]
Milli-Q spiked water (10 μg/l), 20°C, dark	7.58	[5]
Milli-Q spiked water (10 μg/l), 35°C, Suntest light	2.95	[5]
Milli-Q spiked water (10 μg/l), 35°C, Suntest light, 10 mg/l humic acid	1.73	[5]
Buffered distilled water (50 mg/l), 28°C	2.50	[6]
Alkaline solution (400 mg/l), 106.5°C	1.88	[7]
Lake water from Northeast Georgia (0.5 mg/l), 25°C	6.02	[8]
Lake and water samples from Moscow region (0.1 mg/l), 17–22°C	1.04	[9]

favor propanil degradation as it was confirmed by the experiments performed under controlled laboratory conditions. Table 4.1 compares propanil half-lives obtained for different water samples [5–9]. For samples with organic matter with a fertilizer addition (urea) and pH 8, quite similar to the conditions of the Ebre rice crop fields (Tarragona, Spain), there was a close similarity among the herbicide half-lives. However we should point out that most of the experiments were performed with much higher analyte concentrations as compared to this work [5], which unfortunately makes difficult the comparison of propanil half-lives.

We should finally add that the study on the degradation of propanil in rice crop fields was performed with automated on-line (SPE) methods followed by LC-MS, indicating that with appropriate analytical techniques it is possible to follow pesticide degradation in the field and to make controlled experiments in the laboratory at concentrations as low as the concentrations used in the field. Another important issue of this work was that dichloroaniline (DCA) was unequivocally confirmed in the rice crop field waters and flooded soils, and this is a very important issue showing how pesticide metabolites are being formed under real environmental conditions. In this specific case toxicity is of importance too, since dichloroaniline is known to be more toxic tan propanil. Concentrations of the herbicide propanil in water samples varied from 1.9 to 55.9 μg/l. Propanil degraded very rapidly to DCA and high concentrations of this product were found, varying from 16.5 to 470 μg/l in water and 119 ± 22 μg/kg in soil samples. No detectable DCA (<0.001%) was found in the applied pesticide formulation, indicating that DCA formation took place after propanil application. These field results compared favorably with laboratory experiments showing that humic interactions had a strong influence on the pesticide degradation. The half-lives under real conditions for propanil and DCA, calculated using a first-order decay, were 1.2 and 1.6 days, respectively. The results found in the Ebre delta area match those reported from the Yazoo River basin, at the Mississippi [10]. 3,4 DCA was detected at different sites after Spring time with concentrations much higher than those of propanil, reaching values of 0.9 ppb of DCA versus 0.1 ppb of propanil. The half life of propanil was also estimated at 1 day, indicating a similar behavior between the Mississippi River basin and Ebre delta area.

The degradation of propanil under real environmental conditions is a good example of the different advances related to pesticide analysis that are reported in this chapter. First, automated on-line SPE with polymeric material and followed by LC-APCI-MS was used. This implies the use of advanced sample handling and analytical systems that permitted one to obtain high sensitivity and broad environmental information about the degradation of propanil. The methodology used permitted the determination of DCA in real environmental samples which points out that by the appropriate use of advanced analytical methods it is possible to make a more correct diagnostic of the environmental situation and consequently the problems of environmental pollution by pesticides are better known.

To summarize, we should add that in this chapter the recent advances in the different methodologies for the analysis of pesticides in water samples based on LC will be briefly discussed. It should be considered that in 1997 a compilation of the different methodologies was already published [1]. In principle all pesticides are LC amenable, but not all them can be directly or easily detectable with common detectors. In this chapter recommendations on the different analytical methods for pesticides using LC will be reported and for each group of pesticides the most appropriate detection system will be recommended.

4.2 SAMPLE HANDLING (EXTRACTION AND CLEAN-UP)

From the different aspects of sample handling of pesticides some examples about the use of different techniques will be reported. As mentioned earlier, a previous book published in 1997 [1] contains a comprehensive compilation of sample handling methodologies for pesticides in water matrices. Here new additions will be reported. This reflects the authors point of view about different novel or improved approaches for sample handling, including isolation and clean-up of pesticides. Among the different examples reported here, the novel use of Empore disk – by employing SAX disk and double disk approach – the use of immunosorbent for trace enrichment and clean-up of pesticides and some ideas about the future of sample handling of pesticides, including the use of molecular imprints and also automated on-line methods will be reported.

4.2.1 New developments using Empore disks

In 1990 the Empore disk was introduced as a new method in sample preparation for removal of organic contaminants from water and other aqueous samples [1,11,12]. The disks consist of 8–10 μm octyldecyl silica particles that are embedded in a matrix of Teflon, where approximately 10% of the disk by weight is Teflon. The remainder of the disk is the C18 particle. Initially the disk was 47 mm in diameter and contained 500 mg of packing material. This disk was used for the rapid isolation of organic contaminants from a 1-l water sample. It was found that 1 l of water can be passed through the disk in less than 15 min with effective recovery of many pesticides [11]. In 1994, other chemistries of the solid phase were added including styrene divinylbenzene (SDB), cation exchange, and anion exchange, thus permitting the effective isolation of phenolic and other polar analytes [13].

The use of C18 cartridges has been widespread over the past decade for many types of organic contaminants in water [14]. However, there have been few applications of anion

exchange SPE for the isolation of organic compounds. The reasons for his are: the anion exchange SPE isolates organic compounds by the anion exchange mechanism; therefore, the molecule must contain either a cation or anion exchange site. Secondly, inorganic substances present in the water samples also compete for the anion exchange site and will lower the recovery of organic substances in the water sample. Thirdly, the rate of anion exchange on 40–60 μm particles is considerably lower than the rate of adsorption of organic molecules onto a C18 adsorbent. For these reasons, there have been limited applications of the use of anion exchange cartridges for the isolation of organic substances from water.

Empore disks are an alternative to LLE and SPE cartridges. According to Field [16] using the so-called in-vial elution by placing the disks directly into autosampler vials filled with a small volume (2 ml) of solvent, and involving in many cases derivatization is a great advantage of the use of Empore disks. This method has been used for the quantitative determination of acidic herbicides and their metabolites in surface water and for the analysis of diuron and its metabolites in ground water samples [15].

Since the introduction of the Empore strong anion exchange disks in 1994, there have been several applications for the isolation of negatively charged pesticide degradation products on the disks [15,17]. In these studies the capacity of the SAX disk was sufficient to isolate the negatively charged pesticide dacthal from ground water. The disk was found to have high capacity for the doubly charged dacthal metabolites and a rapid rate of adsorption with the 8–10 μm particle size. Apparently, the inorganic ions in the ground water sample had a much lower selectivity for the SAX disk; thus, the Empore SAX disk was an effective solid phase adsorbent for negatively charged species that contained carboxylic groups, which shows that the SAX disk could be an effective sorbent for the isolation of traces of organic contaminants in water samples.

From the work of Field and others, it was realized that the rapid and effective anion exchange capacity of the disk could also be used to remove trace impurities of humic substances which are present in surface and ground water samples and soil extracts. Advantage was taken of the high selectivity of the anion exchange disk for humic substances; thus, they can be effectively removed from water samples during trace enrichment of pesticides from water or soil extracts. The concept of layering the disk is used to first remove the humic impurities on the SAX disk with the isolation of pesticides onto the lower disk that consists of C18. The concept of stacking adsorbents for trace enrichment was first introduced in the early 1980s with XAD adsorbents. In these earlier examples both anion exchange and reversed phase were used to isolate both natural and contaminant organic compounds from water [18].

In a recent paper [19], it was shown for the first time that the Empore disks may be stacked for the effective removal of both trace humic impurities and pesticides. In this way, humic substances were effectively removed by the SAX disk and are not eluted with organic solvents used for isolation of pesticides. In the same paper it was shown that pesticides may be isolated not only on the C18 lower adsorbent but also on the SAX disk.

Fig. 4.1 demonstrates the concept of double-disk solid phase extraction. As the disks are only 0.5 mm in thickness, they may be easily stacked for sample application. This figure shows how the anion exchange disk is placed above the reversed-phase C18 disk. Due to the convenience of the disks they may be readily stacked and eluted either together or separately as needed. The same vacuum apparatus that is used for a single disk may also be
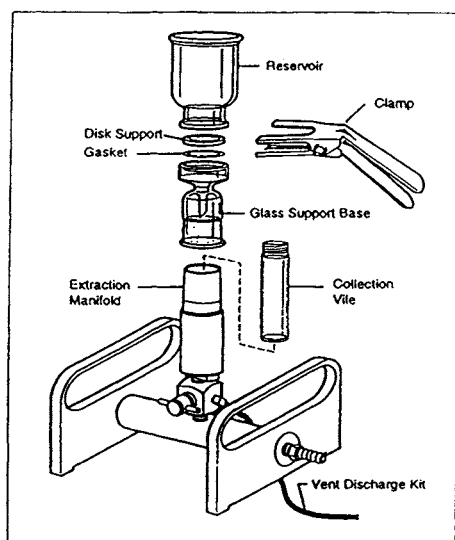
Fig. 4.1. Double disk solid phase extraction (DD-SPE) apparatus.

used for the double disks without leaking or handling problems. The two disks may be conditioned at the same time and eluted simultaneously in order to use the minimum amount of solvent.

Table 4.2 shows the recovery of the phenylurea and triazine herbicides from a 500-ml water sample using the DD-SPE system described in Fig. 4.1. The phenylureas were present at 1 μg/l and were recovered at an average of 89 $\pm$ 9%. These data indicate that the combination of the two disks results in efficient sorption and recovery of the phenylurea herbicides at the trace levels that occur in natural surface and ground water samples. Table 4.1 also shows the recovery data for the triazine herbicides onto the DD-SPE disk. All the triazines studied presented high recoveries indicating a good performance of the double disk methodology for this class of compound, with the exception of deisopropylatrazine which is the most polar one and had a breakthrough lower than 500 ml.

Table 4.3 shows the recovery of the same compounds on the C18 column only, also using a 500-ml water sample at concentrations of 1 μg/l. The average recovery was 97 $\pm$ 8% with the exception of the most polar herbicides, di-demethylfluometuron and deisopropylatrazine. Again these data suggest that the disks are effective at both the sorption and recovery of the herbicides at trace levels.

Table 4.4 shows the recovery of the same herbicides from the strong anion exchange disk. Surprisingly, the SAX disk has some capacity for the herbicides based on a-500 ml

TABLE 4.2

RECOVERIES OF EXTRACTION (%) OF HERBICIDE 7.1. THE RELATIVE STANDARD DEVIATION (RSD) VARIED BETWEEN 3 AND 12% (*n* = 3)

Compound	% Recovery
Di-demethylfluometuron	80
Demethylfluometuron	85
Demethyldiuron	75
Chlorotoluron	101
Fluometuron	95
Isoproturon	99
Diuron	90
Linuron	90
Diflubenzuron	85
Deisopropylatrazine	25
Deethylatrazine	85
Simazine	110
Atrazine	109
Propazine	101
Terbuthylazine	104

water sample at 1 μg/l. The recovery was 47 ± 15% for the phenylurea herbicides. It is important to note that for di-demethylfluometuron the recovery onto the SAX disk was higher than that obtained with the C18 disk. On the other hand, for triazine metabolites, the recoveries obtained with the SAX disk were poor as compared with those obtained with

TABLE 4.3

RECOVERIES OF EXTRACTION (%) OF HERBICIDES FROM 500 ml TAP WATER SAMPLE SPIKED AT 1 μg/l ONTO C18 DISKS. THE RSD VARIED BETWEEN 1 AND 7% (*n* = 3)

Compound	% Recovery
Di-demethylfluometuron	50
Demethylfluometuron	97
Demethyldiuron	79
Chlorotoluron	99
Fluometuron	97
Isoproturon	98
Diuron	108
Linuron	92
Diflubenzuron	82
Deisopropylatrazine	23
Deethylatrazine	83
Simazine	115
Atrazine	110
Propazine	107
Terbuthylazine	102

TABLE 4.4

INDIVIDUAL DISK RECOVERIES OF EXTRACTION (%) OF HERBICIDES FROM 500 ml TAP WATER SAMPLE SPIKED AT 1 μg/l ONTO SAX AND C18 DISKS. THE RSD VARIED BETWEEN 2 AND 10% ($n = 3$)

Compound	% Recovery onto single SAX	% Recovery of the eluate from the SAX disk onto C18 disk	Total recovery (SAX + C18)
Di-demethylfluometuron	65	12	77
Demethylfluometuron	53	33	86
Demethyldiuron	70	20	90
Chlorotoluron	57	41	98
Fluometuron	19	86	105
Isoproturon	54	41	95
Diuron	48	52	100
Linuron	68	35	103
Diflubenzuron	25	65	90
Deisopropylatrazine	4	27	31
Deethylatrazine	10	86	96
Simazine	62	55	117
Atrazine	90	22	113
Propazine	97	12	109
Terbuthylazine	96	6	102

C18 disks. As it can be observed in Table 4.4, the retention of the triazines onto the SAX disks is related to the hydrophobicity of the molecule, indicating a strong affinity of the disk for the less polar triazines, as in a C18 disk. Table 4.4 also shows the recoveries of the herbicides on the second disk, the C18 disk, after its elution separately. The recovery of each of the compounds adds up to 98 ± 4%. This result confirms the recovery of the SAX disk for each of the herbicides under study.

The recovery of the SAX disk was initially quite surprising because the herbicides studied are not negatively charged species; thus, their removal by the SAX disk is occurring from another mechanism of sorption. In a recent paper [19] it was shown that the surface of the SAX disk is quite hydrophilic because of the sorbed water layers around each of the positively charged sites and the ordered water that exists around each of those sites. This simple model suggests that there are equal distances among sites, which of course may not be entirely accurate, but it does give us a back-of-the-envelope idea about the approximate distances between sites and the possibility of hydrophobic zones on the styrene divinylbenzene matrix of the SAX disk.

Next one must consider the polar interactions that are occurring on the surface of the disk at the ion exchange sites. The sites consist of amine groups both of strong (that is continually positively charged) and weak sites (may be charged at acid pH). The strong sites are the fixed ion exchange sites of the disk. The weak sites may share hydrogen atoms and hydrogen bond to the nitrogen atoms of the phenylurea herbicides (see Fig. 4.2). This is a possible mechanism of interaction that may occur on the disk. A comparison of the
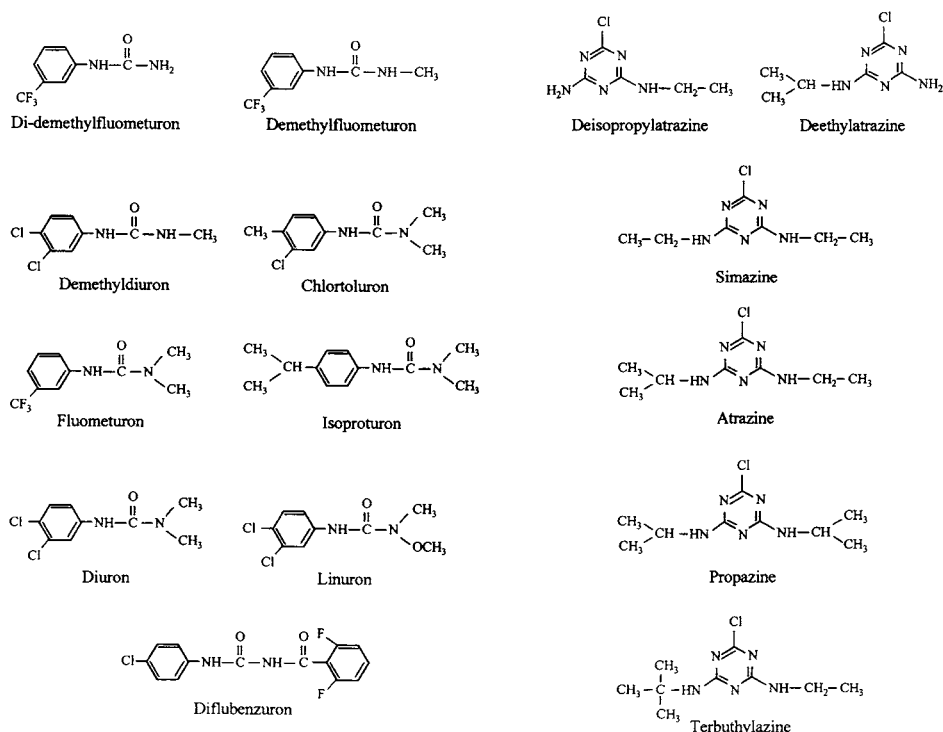
Fig. 4.2. Chemical structures of selected pesticides.

percentage recoveries of each of the herbicides may give more insight into which of the two mechanisms are occurring on the SAX disk.

For example, Table 4.5 shows the recovery of a double SAX disk, instead of the SAX and C18 disk for some of the phenylurea herbicides. Only fluometuron and diflubenzuron had poor recoveries on the double SAX disk, presumably this is caused by a much weaker hydrogen bonding interaction to the polar sites. This conclusion is reached from the fact that the demethyl metabolite of fluometuron has a greater capacity than the parent compound, which is the most hydrophobic of the two compounds. The demethylfluometuron has an extra hydrogen atom present on the amide nitrogen and is more susceptible to the hydrogen bonding mechanism (see Fig. 4.2).

Furthermore, diuron and linuron also have a greater capacity than fluometuron. One reason for this may be that the CF3 group of fluometuron is less electron withdrawing than the chlorine substituents of diuron and linuron. The effect of the electron withdrawing is to increase the acidity of the amide proton of the phenylurea which increases its ability to hydrogen bond to the polar sites Overall, this paper [19] reported the concept and application of the use double disk-SPE and that the use of SAX and/or the combination of SAX C18 permitted an effective isolation and clean-up of several pesticides (see Fig. 4.3) from complex water matrices. This indicates also that the proper use of relatively old materials, like SAX and C18, permits and elegant way of isolation of pesticides thus opening a new window to sample handling.

TABLE 4.5

RECOVERIES OF EXTRACTION (%) OF HERBICIDES FROM 500 ml TAP WATER SAMPLE SPIKED AT 1 µg/l USING THE DD-SPE DESCRIBED IN Fig. 4.1 WITH TWO SAX DISKS. THE RSD VARIED BETWEEN 4 AND 14% ($n = 3$)

Compound	% Recovery
Demethylfluometuron	105
Chlorotoluron	103
Fluometuron	49
Isoproturon	103
Diuron	106
Linuron	117
Diflubenzuron	51

4.2.2 Immunosorbents

In the last few years, a growing interest has been demonstrated in employing the highly selective analyte–antibody interactions achieved by immunosorbents [1,20–24]. In the immunosorbent, the antibody is immobilized onto a silica support and used as an affinity ligand to extract the target analyte and other compounds with similar structures from the aqueous sample. In this way, any material not recognized by the antibody is not retained in the immunosorbent while the target analyte remains bounded to the antibody leading to a high selectivity. The development and the evaluation of two immunosorbents for the selective trace solid phase extraction of phenylurea and triazine herbicides have been presented [20,21]. Other immunosorbents have been described in the literature for the analysis of single pesticides [25–29], like the analysis of carbendazim in soil and lake water by immunoaffinity extraction coupled to LC-MS [28,29]. Correlation of the values obtained by on-line immunoaffinity column followed by LC-DAD and LC-MS with ELISA demonstrated that the trace determination of carbendazim in different types of water samples like creek, fountain and pond water was feasible [29]. Another approach reported in the literature was the use of mixed immunosorbents containing anti-isoproturon and anti-chlortoluron antibodies for the trace enrichment of several phenylureas in water matrices [30]. Such mixed immunosorbents were applied to the trace determination of phenylureas in tap water samples by using on-line SPE enrichment followed by LC-DAD [30].

Although most of the applications reported in the environmental area refer to pesticides, few were reported on other analytes like polycyclic aromatic hydrocarbons in certified reference materials using LC-DAD [24] and on BTEX (benzene, toluene, ethylbenzene and xylene isomers) compounds in complex sample matrices by on-line immunoaffinity followed by LC-membrane introduction mass spectrometry (MIMS) [31].

Many times, sample preparation procedures involve numerous steps. For soil samples, the co-extraction of chemical interferents necessitates a step in the procedure that isolates the analyte from the other components before the final analysis by gas (GC) or liquid chromatography (LC). Immunosorbents have been applied for the clean-up of river water and soil samples followed by liquid chromatography with diode array detection (LC-DAD) [22]. Using immunosorbents, extraction, trace enrichment and clean-up are accom-

plished in one step when analyzing surface waters. So that, chromatograms present a clear baseline allowing the determination and quantification at the 0.01 μg/l level.
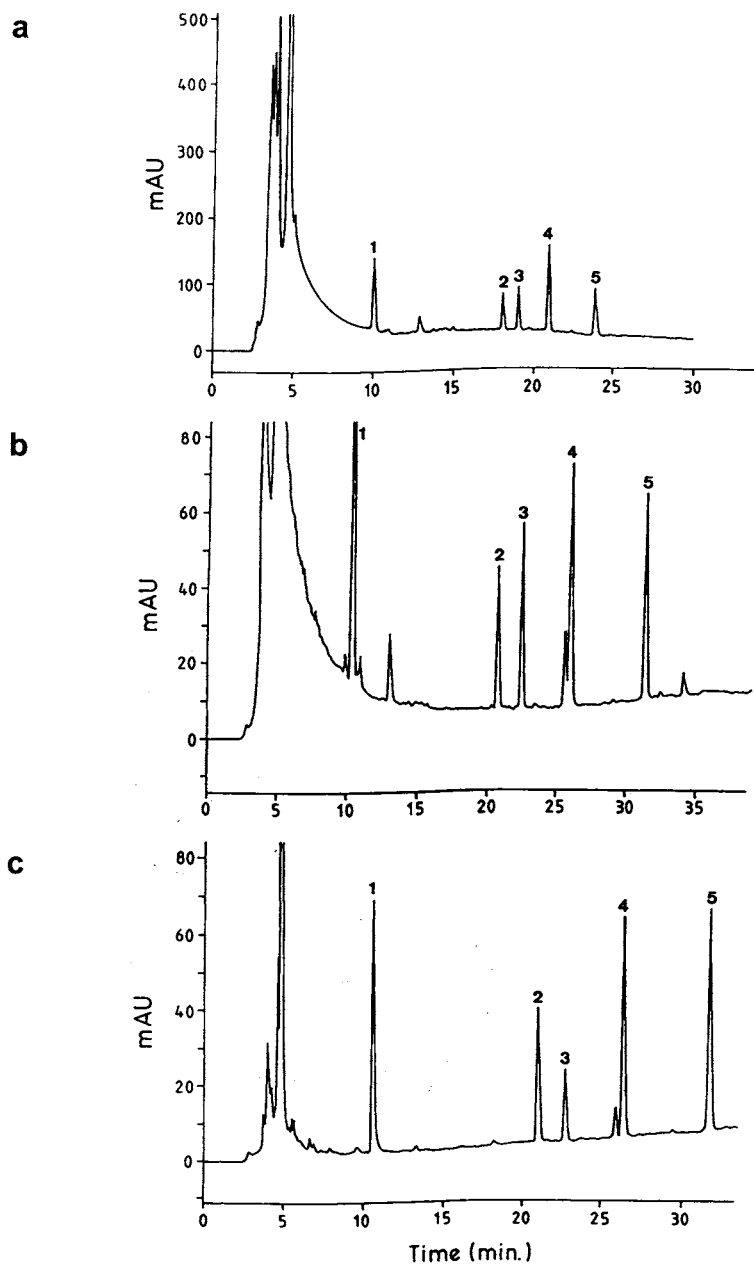
Fig. 4.3. LC/DAD chromatogram of tap water sample spiked with phenylurea herbicides and processed with C18 only (a) and with DD-SPE using SAX and C18 (b), and DD-SPE using SAX and SAX (c). Peak numbers: (1) 2,4-D; (2) demethylfluometuron; (3) fluometuron; (4) diuron and (5) linuron.

On-line immunosorbent coupling to LC-MS has been applied to the determination of several pesticides in environmental samples in a recent paper [32]. By performing this approach, a selective extraction method can be combined with a highly sensitive detection system such as the use of LC-MS with selected ion monitoring. The coupling of such a selective sorbent together with the high sensitivity achieved by LC-APCI-MS allows for a determination of organic pollutants at the low ng/l level using small preconcentration sample volumes. The objectives were [32]: (i) to develop a new analytical method combining immunosorbents on-line with liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC-APCI-MS) for the determination of several pesticides in environmental matrices, (ii) to study the extraction efficiency of triazine and phenylurea pesticides upon anti-atrazine and anti-chlortoluron immunosorbents, respectively, after the preconcentration of ground water samples, (iii) to analyze several sediments and sea water samples to prove the feasibility of such an on-line methodology.

The affinity of the immunosorbents for compounds other than the antigen is achieved due to the similarity in the chemical structures of compounds in the same family of pesticides. This is the case of the anti-atrazine and anti-chlortoluron immunosorbents evaluated in this work [32]; triazines and phenylureas with similar structures to atrazine and chlortoluron, respectively, can be retained in the immunosorbent as their affinity for selective antibodies is succeeded (see Fig. 4.2).

Table 4.6 presents the recoveries of extraction obtained for all the pesticides analyzed after their extraction in 20 ml of ground water spiked at 0.02 μg/l on the anti-atrazine and anti-chlortoluron immunosorbents. For triazine pesticides high recoveries are obtained, ranging from 86 to 103%, with the exception of deisopropylatrazine which was not retained at all in the anti-atrazine immunosorbent. This compound shows a better recovery

TABLE 4.6

RECOVERIES OF EXTRACTION (%) AND REPEATABILITY (RSD AMONG REPLICATES, $n = 5$) OBTAINED AFTER THE PERCOLATION OF 20 ml OF GROUND WATER SPIKED AT 0.2 μg/l WITH A MIXTURE OF TRIAZINES AND PHENYLUREAS THROUGH THE ANTI-ATRAZINE AND ANTI-CHLORTOLURON IMMUNOSORBENT, RESPECTIVELY

Compound	Recoveries (%)	Repeatability (%)
Deisopropylatrazine	0	–
Deethylatrazine	87	10
Simazine	89	1
Atrazine	102	3
Deuterated atrazine	103	4
Propazine	97	5
Terbuthylazine	101	4
Irgarol	86	11
Monuron	80	4
Chlortoluron	90	6
Isoproturon	90	8
Diuron	91	4
Linuron	88	4
Diflubenzuron	42	2

upon anti-simazine immunosorbent rather than upon anti-atrazine immunosorbent, due to the presence of the ethyl group in the molecular moiety. Simazine contains two ethyl groups in its structure, then an immunosorbent containing this antibody can recognize a similar compound; such is the case with deisopropylatrazine. On the other hand, anti-atrazine immunosorbents are expected to trap those triazines that contain an isopropyl group in their structure. For phenylurea pesticides the recoveries ranged from 42 to 91% showing good affinity of the immunosorbents except in the case of diflubenzuron which gave the lowest recovery. In another work [23] the recoveries of phenylurea pesticides upon anti-isoproturon immunosorbents were studied showing low recoveries for chlortoluron and linuron herbicides. For that reason, the performance of anti-chlortoluron immunosorbent together with mass spectrometry detection was evaluated and compared to that from the previous work. Chlortoluron presents a disubstituted phenyl ring, so that, anti-chlortoluron immunosorbent is more suitable for trapping those phenylureas containing disubstituted phenyl rings in their chemical structure such as the case of chlortoluron, diuron and linuron. Although monuron presents a monosubstituted ring it is also retained on anti-chlortoluron immunosorbents due to the presence of the dimethylamide group in the chemical structure. Incomplete recoveries may be due either to the low affinity presented by the antibodies towards the analytes or to the overloading of the capacity of the immunosorbent when related compounds are competing for the same binding sites. In the first case, the recovery is limited by the retention factor of the analyte by the immunosorbent whereas in the second case, a decrease in the competition process will represent an improvement in the recovery.

Repeatability of the method was calculated from five independent extractions of triazine and phenylurea pesticides from ground water samples on anti-atrazine and anti-chlortoluron immunosorbents, respectively. The repeatability ranged from 1 to 11% indicating good performance of the method developed in this work. One advantage of automation in an on-line preconcentration is that more reproducible results are expected, provided that the manipulation of the samples is avoided as compared with an off-line methodology. Moreover, the use of internal standards represents an improvement in the precision of measurements when performing the quantitation of analytes by LC-MS since the signal variations of this system are quite common. Variations in the solvent pumps and plugging problems encountered in the source are the main causes of the lack of reproducibility achieved in many measurements. The use of internal standards, either deuterated or not, is therefore, a good analytical quality tool when quantifying with LC-MS.

Atrazine, deethylatrazine and linuron were found in the sediment samples analyzed with the methodology developed in this work. The herbicides atrazine and linuron were used in the rice crop fields of the Ebre Delta area in amounts of 1 ton/year of active compound during 1990–1991. These herbicides are used for weed control of corn, wheat, barley and sorghum. In Table 4.7 the environmental concentrations found in all the sediments analyzed by LC-APCI-MS are presented. Concentrations of atrazine are lower than those for linuron and it can be explained by the difference in the respective K_{OC}. The value of this parameter for atrazine is 160 whereas for linuron it is 400, indicating that this compound is more likely to remain in the sediment than in the water.

Deethylatrazine was the major metabolite of atrazine found in the sediment samples. The degradation of the herbicide atrazine in soils has lead to numerous investigations. Dealkylation of atrazine is the most significant biotic degradation pathway for atrazine in

TABLE 4.7

CONCENTRATION VALUES (μg/kg) OF THE PESTICIDES ANALYZED IN SEDIMENT SAMPLES AND IN SEA WATER SAMPLES (μg/l) AFTER THEIR ANALYSIS BY SOLID-PHASE-IMMUNOSORBENT-EXTRACTION FOLLOWED BY LC-APCI-MS. EXPERIMENTAL CONDITIONS AS DESCRIBED IN THE TEXT

Samples	Compound					
	Deethylatrazine	Atrazine	Linuron	Diuron	Irgarol	DAR[c]
Sediment 1[a]	19.5	33.6	–[d]	–	–	0.58
Sediment 2	6.5	19.2	–	–	–	0.34
Sediment 3	15.4	39.2	–	–	–	0.39
Sediment 4	–	–	139.0	–	–	
Sediment 5	–	–	59.2	–	–	
Sea water 1[b]	–	–	–	0.04	0.04	
Sea water 2	–	–	–	0.02	0.03	

[a] Sediment samples were collected in the Ebre Delta area during 1990–1991.
[b] Sea water samples were collected in the Masnou area during 1996.
[c] DAR, deethylatrazine:atrazine ratio.
[d] –, not detected.

soil environments. The conversion of atrazine to its main metabolite, deethylatrazine, is primarily due to the result of metabolic activity of soil bacteria and fungi and is the major degradation product of atrazine in soils. The DAR (deethylatrazine:atrazine ratio) is a valid indicator of soil-mediated transport of atrazine to an aquifer [14,32]. A DAR of unity or greater may be an indicator of non-point-source contamination of an aquifer whereas a small DAR value may be an indicator of point-source contamination of an aquifer. In the case of non-point-source contamination, as atrazine is normally stored in sediments, soil microorganisms can convert significant quantities of atrazine to deethylatrazine, thereby increasing the DAR. On the other hand, point-source contamination resulting from a direct entry of atrazine into an aquifer, would not involve prolonged contact of the applied atrazine with soil microorganisms and then the concentration of deethylatrazine would be lower than the concentration of atrazine. In this case the DAR value would be smaller than unity. In the present study, a DAR value smaller than unity was obtained (see Table 4.7), thus indicating a point-source contamination of the aquifer that contained the sediment samples analyzed in this work. This can be easily explained if we take into account that samples were collected a few months after the application period of the pesticides and that atrazine is currently applied over this area.

As can be seen in Fig. 4.4, the chromatograms obtained from the sediment extracts analyzed after their preconcentration through the anti-atrazine immunosorbent presented a clear baseline and the compounds could be easily identified and detected. The selectivity of the preconcentration through the immunosorbent is so high that small sample volumes, such as 20 ml, can be used as compared with those normally analyzed by non-selective sorbents. Normally, a clean-up step before the GC or HPLC detection is needed due to the complex matrix present in these kinds of samples. However, since selective interactions by the antibodies are achieved using immunosorbents, this additional step is avoided which
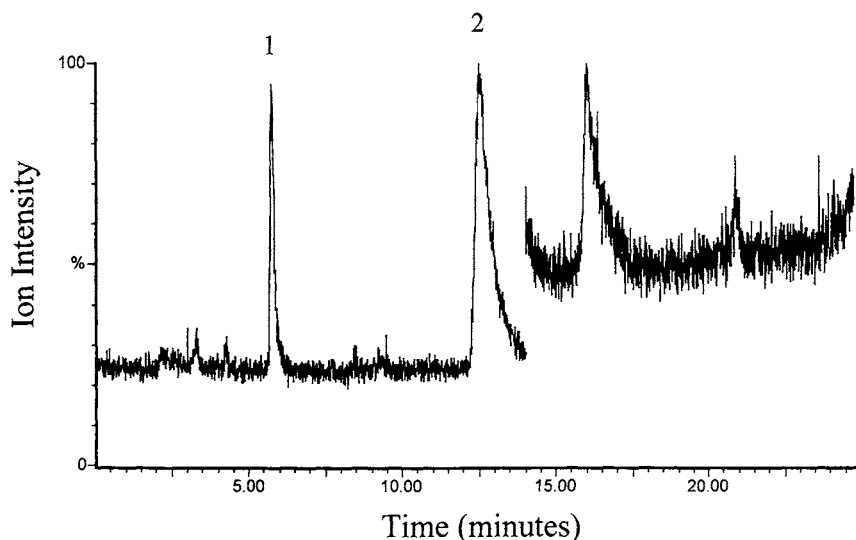
Fig. 4.4. On-line solid phase extraction of 20 ml of water containing the extract of a sediment sample through an anti-atrazine immunosorbent followed by LC-APCI-MS in positive ion (PI) mode of operation and under time-schedule SIM-PI conditions. Analytical conditions as described in the text. Peak numbers: (1) deethylatrazine; (2) atrazine + deuterated atrazine.

allows one to obtain lower coefficients of variation in the measurements. Blanks of sediment samples were also preconcentrated through both immunosorbents and analyzed by LC-APCI-MS under SIM and SCAN conditions showing no significant interferences with the pesticides studied.

Sea water samples were preconcentrated on the anti-atrazine and anti-chlortoluron immunosorbents and analyzed by LC-APCI-MS under SIM conditions. Irgarol and diuron concentrations in the ppb level (see Table 4.7) were found in the two sea water samples collected from a marina and were well correlated with those analyzed in the previous study carried out with these samples [33]. Both compounds are used in antifouling paints as a biocide agent in substitution to the tributyltin (TBT) and copper-based agents. These compounds are used in tin-free antifouling paint formulations which are mainly based on copper and zinc metal oxides. The herbicides are added in order to inhibit the primary growth of copper resistant fouling organisms such as algal slimes and the growth of seaweeds on sea boats. Irgarol and diuron have a good recovery on anti-atrazine and anti-chlortoluron immunosorbents, respectively, therefore their feasibility to be extracted from a sea water sample is accomplished by means of the selective interaction between the antibodies and the pesticides. This result confirms again the high selectivity of the immunosorbent for the compounds present in any type of water.

Overall immunosorbents are an excellent alternative for sample handling of pesticides from natural water samples. A question remains about the commercialization and future use of immunsorbents on a large scale. At present, most immunsorbent columns are made by polyclonal antibodies, so it is difficult to assure long-term reproducibility of new columns prepared with polyclonal antibodies. The fact that different rabbits are used

and long-term stability are key issues. There are few projects at European level involving the preparation of immunosorbent columns by using monoclonal antibodies. This will assure the reproducibility of the measurements and consequently the possibility to use such columns for routine applications. At present, in the environmental field, they are mainly used in research laboratories although in food analysis some of them commercially available, e.g. for aflatoxins. Another alternative to the use of immunsorbents is the use of molecular imprints as SPE materials. This will be briefly discussed here.

4.2.3 Molecular imprints

An emerging technology, called molecular imprinting, leads to highly stable synthetic polymers that possess selective molecular recognition properties. Recognition sites within the polymer matrix are complementary to the analyte in the shape and positioning of functional groups. The polymers prepared with synthetic recognition sites exhibit a predetermined selectivity for analyte(s) of interest. The imprint is obtained by arranging polymerizable functional monomers around a template (the analyte). Complexes are then formed through molecular interactions between the analyte and the monomer precursors. The complexes are assembled in the liquid phase and fixed by crosslinking polymerization. Removal of the template from the resulting polymer matrix creates vacant recognition sites that exhibit affinity for the analyte. After removal of the this template molecule, the polymer can be used as a selective binding medium for the template (analyte) or structurally related compounds. The mechanism by which these polymers specifically bind the template and related ligands are attributed to the formation of functional groups in a specific arrangement within the polymer that corresponds to the template and to the presence of shape-selective cavities. Some of these polymers have selectivities and affinity constants, comparable with naturally occurring recognition systems such as monoclonal antibodies, which make them especially suitable as constituents for enhancing selectivity in SPE.

The potential of MIPs is high [34–36], since they show physical robustness, high mechanical strength, resistance to elevated temperatures and pressures, and stability in the presence of extremes of acids, bases, metal ions and organic solvents, features that are favorable for routine analysis [37]. The practical application of MIPs for environmental analysis is very limited. MIPs in analytical procedures to date, encompass sample preparation, analytical separation and detection techniques, as depicted in Fig. 4.5. During the initial step of an analysis, the sample is treated in order to allow analytical separation and detection with a minimum risk for artifact formation. Failure to do so will lead to erroneous results due to, e.g. degradation of analyte, decreased performance of the chromatographic column, adsorption, or clogging. One approach is to implement MIPs as chromatographic material in solid .phase extraction (MIP-SPE). The basic concept is that the chromatographic parameters are tuned such that the MIP-SPE-column traps the analyte, or a group of structurally related compounds, whereas matrix components are not retained, see Fig. 4.5A. After washing these off the MIP-SPE-column, the compound of interest is eluted for further analysis.

A known challenge for MIP-SPE is the presence of remaining template species in the MIP, which reduces the capacity of the column (less binding sites are available for the analyte). In trace analysis, column capacity is not a major concern, however, leakage of

A) Solid Phase Extraction

B) Chiral Separation

C) Affinity Assay
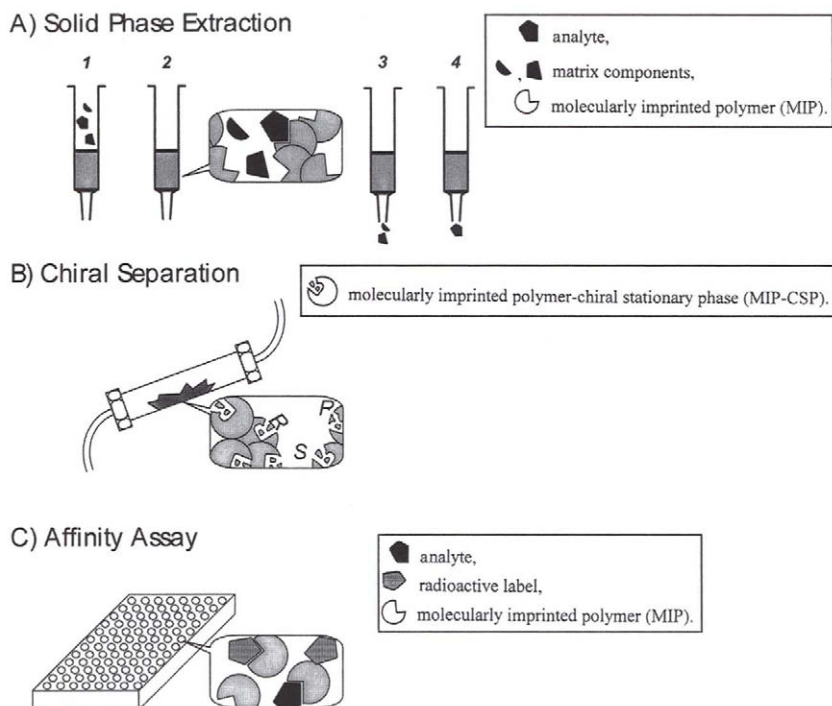
Fig. 4.5. Application of molecularly imprinted polymers in (A) sample pretreatment, (B) analytical separation and (C) detection.

remaining template species will contaminate the sample. In addition to extensive washing of the MIP prior to use, contamination of sample can be circumvented by employing a structural analogue to the analyte during imprinting. When template leaking occurs, the template can be separated from the analyte in the analytical separation step following MIP-SPE.

So far, all applications of MIP-SPE deal with low molecular mass analytes, among them pesticides. For these compounds, antibody-based affinity chromatography for sample treatment can offer similar advantageous features as MIP-SPE. However, the development of antibodies against the compound(s) of interest may prove more difficult than the synthesis of a MIP, which is true for certain very polar pesticides like glyphosate but not for common ones like atrazine, as we have noticed before in this introduction. What MIP offers, in contrast to immunosorbents is its robustness, long-term stability and probably reproducibility with regard to column preparation, which is quite difficult in the case of immunsorbents using polyclonal antibodies. Full compatibility with, or even improved selectivity in, organic solvents makes MIP-SPE an interesting technique especially for hydrophobic analytes.

They are few publications reporting the application of MIP to the trace determination of pesticides [38–40]. Atrazine and 2,4-dichlorophenoxyacetic acid assays using molecular imprinted polymers were developed. Application to the trace determination of various

triazine pesticides in water samples was also reported [40] although the results, at present, need to be improved. In some cases, selectivity is not very high and also other analytes and interferences present in the water matrix can be easily trapped by the SPE material. In this respect this area will be developed in the next coming years since there is interest from different commercial firms and research institutes to carry out further developments in this direction.

4.2.4 SPME

Solid-phase microextraction (SPME) is a novel extraction technique that was first introduced by Pawliszyn [41]. SPME offers some advantages over the more conventional preconcentration methods used for determination of organic compounds in environmental samples, such as LLE or SPE. For instance, it does not require the use of organic solvents, which are needed in both LLE and SPE. In addition to that SPME is simple, fast, easy to automate, portable, inexpensive – much cheaper than SPE – and in general small water volumes are sufficient to extract the analytes of interest.

SPME can be easily coupled to GC and the injection port of the GC can be used for the thermal desorption of analytes from the fiber. When the temperature increases, affinity of the analytes towards the fiber decreases and they are liberated. Usually desorption is achieved in less than 2 min for most of the compounds. SPME-LC has been introduced more recently and it is more complex than SPME-GC because it requires the use of an interface to achieve the desorption of the analytes. Parameters that affect the desorption process in LC such as the selection of the most appropriate solvent and heating of the interface, have been evaluated by some authors [42]. Organic solvents can damage the fiber coating and for this reason not all SPME fibers can be used for LC applications. Fibers for LC involve carbowax divinylbenzene mixtures. A more recent development of SPME-LC, called in-tube SPME, was introduced by Eisert et al. [43]. This application allows an automated SPME-LC system to be developed. In this new SPME design, a piece of open tubular capillary GC column or a piece of a micro-LC capillary column was used as the extracting phase. Only few group of compounds were determined by SPME-LC, like non-ionic surfactants [44], phenylurea and carbamate pesticides [43,45] and polycylic aromatic hydrocarbons [46].

For the analysis of aqueous samples, direct immersion extraction is the most used SPME sampling mode [47–49]. For instance Lee et al. [47] developed a method for determining acidic herbicides in water by SPME followed by GC. It is known that acidic herbicides are very polar compounds not directly amenable by GC. In this paper the so called "in fiber " derivatization method was used. The post derivatization takes place in the fiber following SPME with diazomethane gas procedure combined with GC-MS. Detection limits are in the low ng/l range. The method includes also some degradation products of phenoxy acids like 2,4-dichlorophenol, 2,4,5-trichlorophenol and 4-chloro-3-methylphenol.

The introduction of new SPME fibers has extended the range of applications of SPME to other classes of analytes, but still the technology is basically GC-oriented. The combinations of SPME with LC is more difficult and needs to be developed and improved. Most probably SPME will remain a very useful, routine and automated method followed by GC. It will probably substitute other sample preparation methods like SPE. Another relevant approach, not yet published, would be the combination of immunofibers and/or molecular

imprints coupled to SPME. This will be a very selective option for sample preparation and a technological challenge.

4.2.5 Automated methods

Automated on-line solid phase extraction methods are much more common in Europe compared with the US. This is basically due to the Rhine Basin Program which started in 1987 with the main objective of protecting the waters of the river Rhine from chemical pollutants. The specific objectives of the Program and their consolidation are described in detail by van Hout and Brinkman [50]. In brief, the Program was focused to implement totally automated systems for the on-line monitoring of organic contaminants in water. In the case of pesticides, such monitoring is compulsory due to the toxicity that these compounds cause to the environment, even at low concentrations (0.02–10 µg/l in surface waters). Moreover, local accidents may pose a problem to the environment salubrity [51] and the surveillance of the water quality in a quick and reliable way becomes necessary. In view of the need of automated methods [52], several research groups adhered to the Rhine Basin Program designed the SAMOS system (System for the Automated Monitoring of Organic Pollutants in Surface water), which was applied to determine pesticides of different families in surface waters in different European rivers [53,54]. Automation of SPE is possible both with gas chromatography (GC) [55] or by liquid chromatography (LC) [56,57]. While the former technique permits an excellent selectivity and limits of detection (LOD), the latter can simultaneously analyze pesticides of a larger spectrum of polarities, volatilities and also the thermolabile. Moreover, LC based techniques allow one to trace polar pesticides, like phenylureas, and degradation products in environmental waters, such as the oxo analogues, sulfoxides or their isomers [58,59]. The SAMOS design, as it is now, is made to function without interruption for several days, but it has two weak points from the viewpoint of routine pesticide monitoring in surface waters: the sample has to be carried to the laboratory before it is analyzed and it has to be filtered through 0.45 µm filters to remove suspended particles. The development of a fully automated system was the main objective of the European Project EV5V-CT92-105 entitled ''The development of an automated monitoring system for the determination of pesticides and their conversion products at trace level in environmental waters''. The objective was to develop and test a prototype which permitted the improvement of surface water pesticide analysis with regard to automated analysis. The method presented is designed to be used in situations such as in water depuration plants or water management stations, where a 24-h pesticide survey is necessary in order to assess water quality. Therefore, the main advantages of the new, totally automated system are: (i) it permits coupling on-line the filtration step, storage of the water samples, the preconcentration step and the chromatographic analysis. Coupling all these steps on-line avoids sample manipulation and facilitates unattended sampling and analysis, with the additional advantage that human power and errors are reduced, and (ii) it permits the detection of real-time sporadic episodes of pollution by analyzing river waters.

A modification of the SAMOS system has been performed to fully automate the water sampling, filtration of the water sample, preconcentration and chromatographic analysis [60]. The commercially available SAMOS consists of a preconcentration unit, Prospekt (Spark Holland, The Netherlands) responsible for the SPE step, which is coupled on-line

with a liquid chromatography instrument (HP1090) equipped with a diode array detector (LC-DAD) (Hewlett Packard, Waldbron, Germany). The modifications include: (i) the attachment of a sampling unit, which incorporates a pump and four refrigerated flasks, on-line prior to the preconcentration unit and (ii) a novel filtering device connected on-line with the rest of the system to prevent clogging of the equipment due to the presence of particulate matter. The filtering devise consists of a 50-cm stainless steel cylindrical tube with pores of 10 μm connected to a 190-mm diameter glass fiber filter of 1 μm pores. The scheme of the SAMOS with refrigerated unit that permits the operation under high summer temperatures is shown in Fig. 4.6 and the new filtration system is shown in Fig. 4.7.

The overall system can be installed in laboratories where the river/surface water can be conducted to the instrument. River water is pumped through the filtration devise, and afterwards inside the flasks, which act as sample reservoirs. Every 4 h, an aliquot of 100 ml is preconcentrated onto C18 precolumns of the preconcentration unit and analyzed. The system is programmed in a way that each day each samples are being analyzed, and can be operated unattended for at least 5 days. This configuration permits a satisfactory performance, with the unattended monitoring of surface river water and limits of detection (LOD) at levels of 30–100 ng/l. The system is used in situations where a continuous and unattended monitoring of organic pollutants in surface waters is needed, e.g. water treatment works. (see Fig. 4.8 as an example).

The main improvement with regard to other on-line SPE-LC-DAD methods is the filtration device. In some locations it is not necessary to filter the water sample before preconcentration. However, some rivers or other surface water present high amounts of particulate matter that has to be removed before analysis. On-line systems described up to now only contemplated automated SPE trace enrichment followed by multiresidue analysis, and filtration was performed off-line prior to the analysis of the pesticides. The filtration mechanism proposed is a home-made design, the advantage of which is that percolation of the water through the 1-μm disk filter is done by using the pumps of the sampling unit, so that only the needed amount of water which will be analyzed goes
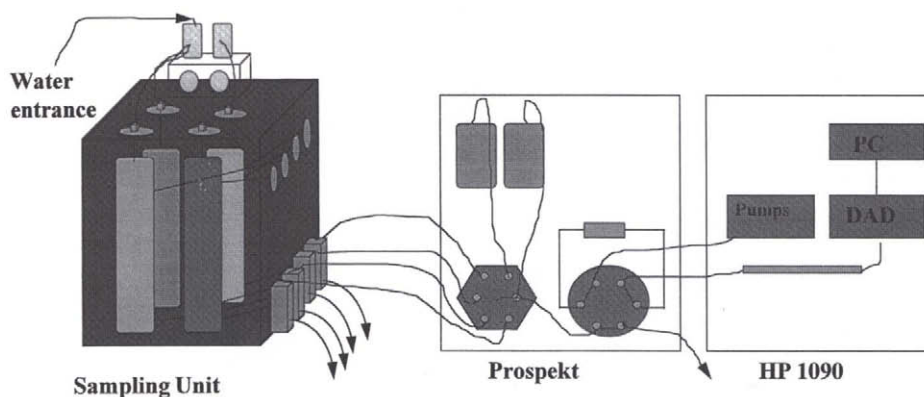
Fig. 4.6. Scheme of the refrigerated unit that was adapted to be used with the conventional SAMOS. It contains four flasks, and the filling and emptying is controlled by a group of valves and pumps, controlled either manually or by the PC
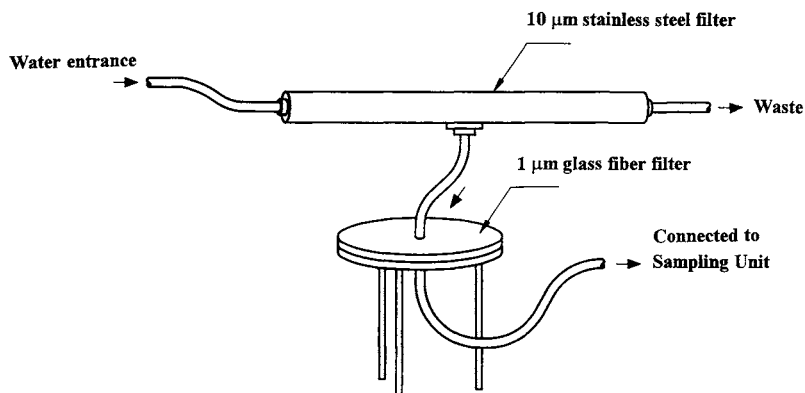
Fig. 4.7. Filtration system design. It consists of a 50-cm long stainless steel cylindrical tube with a 10-μm pore size connected on-line to a 1-μm glass fiber disk, which is connected to the flasks.

through the disk, reducing the possibility of obstruction (see troubleshooting). In normal conditions, the glass filter disks allowed the filtration of 25 l of water where the common turbidity is around 60–100 NTU. Translated to days, the system is suitable for unattended sampling during at least 5 consecutive days, without changing any filter. With that proce-
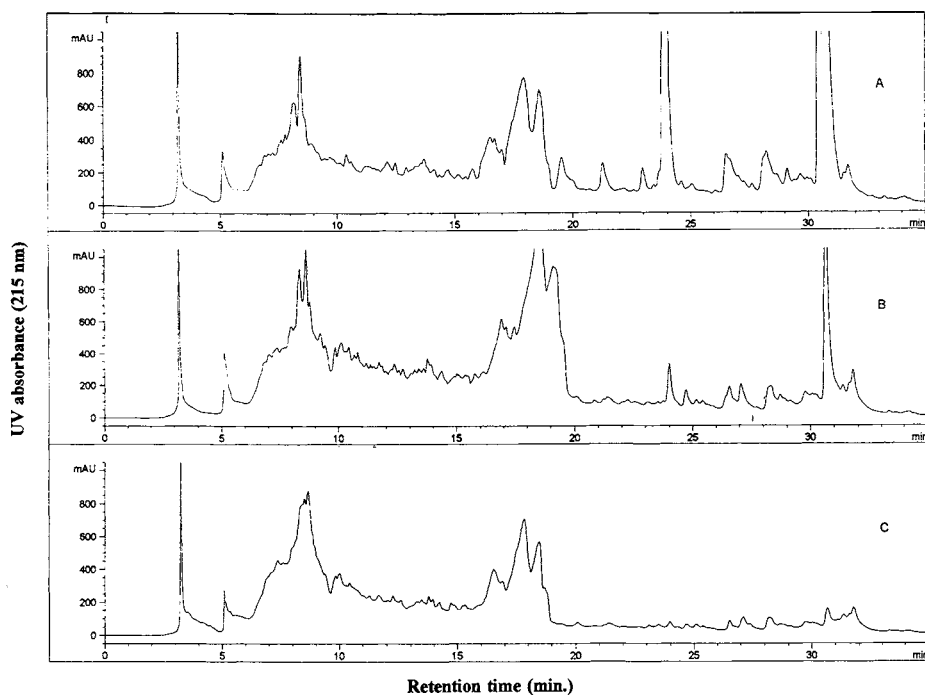
Fig. 4.8. On-line SPE-LC-DAD chromatogram at 215 nm of untreated Llobregat river water samples. Samples were taken at different times on a single day. A = time 0, B = 8 h after A and C = 16 h after A.

dure, 98% of the particles are removed by using the 1 μm filter and the turbidity decreases to 0.33 NTU for waters that had an initial value of 66 NTU.

In a recent paper an automated SPE-LC-DAD system, similar to that of SAMOS, and coupled LC-LC was compared for residue analysis of triazines and their metabolites in water matrices [56]. The advantages and drawbacks of each technique were discussed, concluding that their choice is determined mainly by the problem to be solved (sensitivity required, matrix interferences, number of analytes and samples to be analyzed) as well as the technical possibilities and expertise of the laboratory.

4.2.6 Clean-up methods

In general clean-up methods are not generally needed when ground or drinking water samples are analyzed. When other types of water matrices, like river or estuarine water matrices, are analyzed by SPE methods, then the same SPE column is used as the clean-up method. Clean-up methods are always needed when analyzing soil and food matrices, since in these cases the extract obtained after Soxhlet or other type of extraction like microwave assisted extraction (MAE) or accelerated solvent extraction (ASE) needs further clean-up. From the different clean-up methods currently used, one of the most popular ones is the use of chromatographic columns packed with different materials for a selective clean-up of certain analytes. The clean-up techniques most commonly employed for extracts containing residues of pesticides are liquid–liquid partitioning or adsorption chromatography such as column chromatography and SPE. Many methods require a combination of both.

In a recent paper [61] an interesting example on the use of different clean-up methods for N-methyl carbamate (NMC) pesticides in vegetable extracts was reported. The method has compared several clean-up procedures of extracts containing selected carbamates, by the use of glass columns and SPE cartridges.

Silica, alumina, and Florisil columns and Silanized Celite/charcoal columns were used. In the case of Celite/charcoal columns, Celite was treated with dimethyl-dichlorisilane, and charcoal was purified by acid washing according to the method described in the Pesticide Analysis Manual [62]. One part charcoal and four parts Celite were combined by vigorous mixing and stored in a sealed container. Glass and plastic (hypodermic syringe, capacity 10 ml) columns were packed with this mixture as shown in Fig. 4.9. Other clean-up methods reported in the same paper involved the use of reversed-phase (C8, C18, CN) and normal-phase (silica, CN, NH2)-SPE cartridges. All cartridges contained 500 mg adsorbent. In the case of reversed-phase SPE cartridges non-polar C18, C8, CN cartridges were used. After the bonded phase had been solvated with 15 ml of methanol at 5.0 ml/min flow rate, the cartridge was loaded with 0.5 ml standard solution. The NMCs were eluted with 5.0 ml of each elunt system (ES) in decreasing order of polarity (2.0 ml/min flow rate). The fractions were collected, evaporated to near dryness in a rotary evaporator and concentrated under a stream of anhydrous nitrogen gas. The final residue was then reconstituted with 0.5 ml methanol, filtered and analyzed by HPLC. For normal-phase SPE cartridges polar silica, CN and NH_2 cartridges were conditioned with n-hexane and the clean-up procedures were performed as described above, using 5.0 ml of each eluting solvent in gradual increased polarity. Although sample extraction and partitioning procedures were based on Krause's method [63] some modifications were
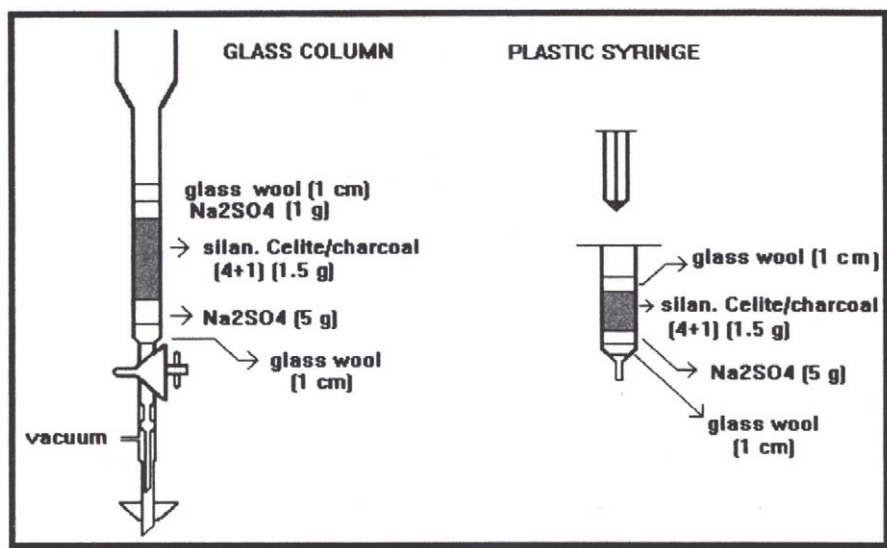
Fig. 4.9. Silanized Celite/charcoal columns used for clean-up of *N*-methylcarbamate insecticides.

included in order to minimize the number of steps and allow the final determination by HPLC/UV detection. The method proposed in the recent work [61] to carbamate analysis is summarized in Fig. 4.10.

Due to the different polarities of the carbamates, the development of a simple and unique clean-up method is very difficult to achieve, especially if more than one compound is simultaneously determined. First, the elution pattern of the carbamates present in standard solutions was studied using chromatographic columns packed with silica, alumina, Florisil and Celite/charcoal and several binary mixtures as eluent solution. According to the results shown in Table 4.8 the best eluent systems (ES) and adsorbents were: methylene chlorine/methanol (99:1), toluene/acetonitrile (75:25), deactivated alumina (4.6%) and silanized Celite/charcoal (4 + 1, plastic column), respectively. Under these conditions, all the pesticides had total recoveries higher than 85%. The ES number 1 (acetone/*n*-hexane, 15:85) resulted in poor recoveries due to its lower polarity and insufficient capacity of elution of polar carbamate. Overall recoveries were improved with low deactivated adsorbents. For example, the 2% deactivated Florisil provided higher recoveries than 4, 6 and 10% deactivated alumina and silica, respectively. Such behavior is due the water present in these adsorbents which can hydrolyze the carbamates, as reported by Bertrand et al. [64]. Florisil activated at 600°C was tested too, but the recoveries were extremely low. Thus, Florisil is a preferential deactivated material for carbamate clean-up because less OH– groups are present on adsorbent surface that enable the efficient retention of the compounds prior elution with the mixture methylene chlorine/methanol (99:1). This results agrees with the observations of Barceló and co-workers [65] about deactivated adsorbent surfaces. Durand and Barceló [66] studied different clean-up methods for the determination of different class of pesticides – including seven carbamate pesticides – from environmental soil samples. They reached satisfactory results using a deactivated
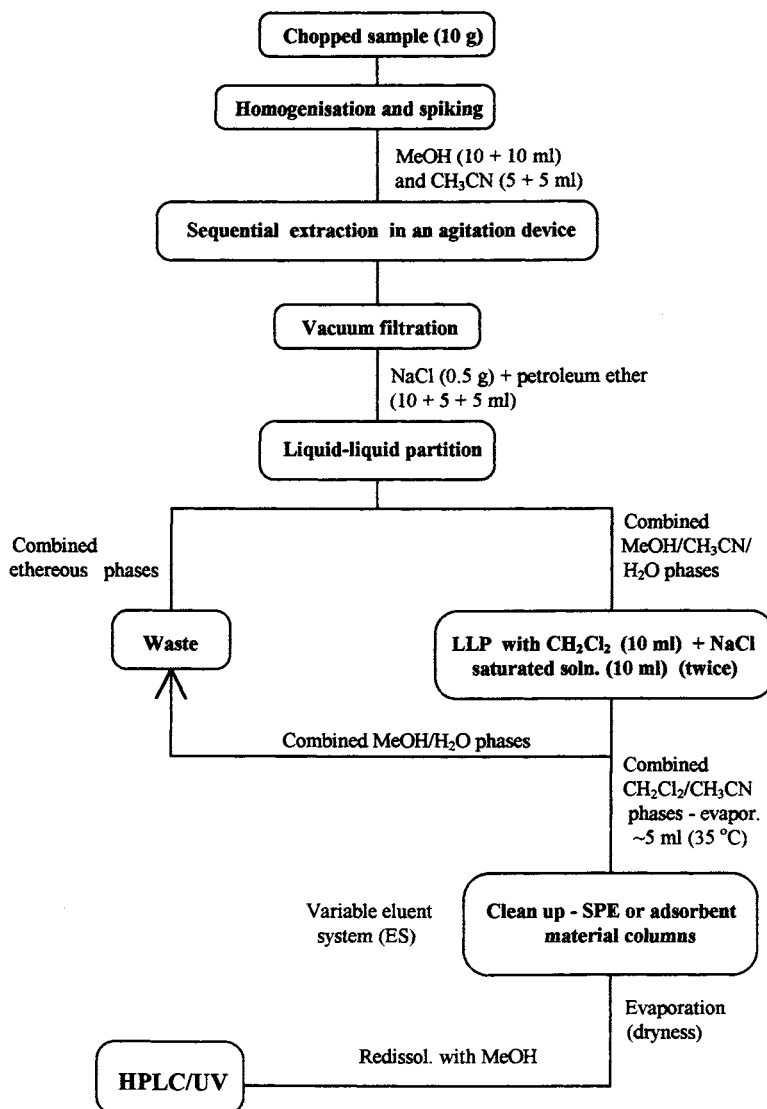
Fig. 4.10. Scheme of extraction and partitioning procedures before HPLC-UV analysis of the selected *N*-methylcarbamate insecticides in potato and carrot samples.

Florisil column combined with a binary mixture acetone/dichloromethane (50:50). Such eluent has polarity similar to the third ES examined in the present study.

The Celite/charcoal mixture was very powerful in retaining the more polar compounds and needed a strong ES like toluene/acetonitrile to elute all the NMCs. Therefore, in extracts where a minimum content of water is present, some emulsion can be formed during elution process. In this case, the third ES (methylene chlorine/methanol, 99:1) is recommended for use without considerable losses of the pesticide recoveries. The advan-

TABLE 4.8

RECOVERY OF THE *N*-METHYLCARBAMATE INSECTICIDES AFTER ELUTION ON ADSORBENT COLUMNS[a]

Adsorbent material	ES	Recovery (%)				
		Methomyl	Aldicarb	Propoxur	Carbofuran	Carbaryl
10% deactivated silica	1	63 (6.5)	67 (3.2)	66 (2.6)	68 (3.8)	37 (5.5)
	2	94 (4.6)	80 (3.4)	79 (2.3)	85 (3.1)	83 (4.0)
	3	ND	91 (2.7)	79 (3.6)	72 (4.2)	18 (5.0)
	4	80 (12.6)	67 (3.5)	52 (2.7)	59 (4.2)	33 (4.8)
4.6% deactivated alumina	1	81 (4.9)	66 (2.6)	77 (3.2)	91 (4.4)	98 (5.6)
	2	81 (4.0)	69 (3.3)	67 (4.1)	72 (5.3)	44 (4.6)
	3	96 (5.5)	94 (2.7)	89 (3.0)	105 (4.0)	89 (5.2)
	4	69 (6.6)	47 (3.6)	69 (5.3)	70 (5.4)	58 (4.2)
2% deactivated florisil	1	51 (6.0)	91 (4.0)	67 (3.7)	81 (4.2)	63 (8.0)
	2	65 (6.2)	73 (2.8)	63 (4.0)	75 (3.5)	54 (5.4)
	3	83 (5.0)	108 (2.5)	101 (3.2)	102 (3.7)	95 (2.8)
	4	85 (12.0)	120 (3.3)	104 (2.9)	109 (4.0)	94 (2.8)
Activated florisil	1	60 (8.0)	79 (2.6)	68 (4.0)	68 (3.6)	48 (3.4)
	2	83 (6.7)	62 (3.0)	75 (3.3)	87 (3.0)	91 (4.0)
	3	99 (7.0)	81 (2.5)	76 (3.0)	87 (4.0)	71 (3.0)
	4	101 (6.5)	90 (3.5)	96 (6.0)	110 (3.5)	108 (4.0)
Silanized Celite/charcoal (syringe)	1	33 (6.2)	85 (2.5)	73 (3.6)	72 (4.0)	40 (3.0)
	2	104 (5.4)	85 (2.6)	60 (2.5)	66 (2.7)	ND
	3	89 (6.0)	92 (2.0)	85 (3.0)	94 (3.2)	97 (2.5)
	4	97 (5.0)	97 (3.5)	86 (5.3)	91 (3.5)	90 (4.6)
Silanized Celite/charcoal (column)	1	8 (4.0)	29 (5.0)	29 (5.0)	39 (5.2)	6 (5.4)
	2	102 (5.5)	98 (2.5)	89 (3.0)	81 (4.5)	21 (6.0)
	3	100 (4.5)	88 (3.2)	87 (4.5)	79 (3.5)	88 (4.3)
	4	105 (3.5)	82 (6.6)	80 (3.5)	92 (2.5)	77 (5.0)

[a] ES, eluant systems: (1) acetone/*n*-hexane (15:85); (2) methylene chloride/*n*-hexane (80:20); (3) methylene chloride/methanol (99:1); (4) toluene/acetonitrile (75:25). Coefficients of variation (%) in brackets ($n = 3$); ND, not-detected.

tage of charcoal is to retain efficiently some co-extractives, for example pigments (xantophyll, chlorophyll, etc.) [62,63]. However, the preparation of this adsorbent mixture is very laborious and time consuming.

After efficiency of the glass columns had been examined, elution on SPE cartridges was studied. Table 4.9 shows that elution procedure in normal-phase cartridges resulted in adequate recoveries (71–120%), especially for cianopropyl- and aminopropyl-bonded cartridges (84–120%) due the similarity of the structures and polarities of these adsorbents and the selected carbamates. The elution protocol proposed in this work was peculiar because it was performed with a small volume (5.0 ml) of each ES in increasing or decreasing polarity according to normal or reversed mode, respectively. In general the eluting mixture it is chosen and the elution is performed in one unique turn after column

TABLE 4.9

RECOVERY OF *N*-METHYLCARBAMATE INSECTICIDES AFTER ELUTION IN SPE CARTRIDGES[a]

Sep-Pak cartridges	Recovery (%)				
	Methomyl	Aldicarb	Propoxur	Carbofuran	Carbaryl
Silica (NP)	71 (4.3)	94 (2.5)	88 (2.0)	92 (2.5)	96 (3.5)
CN (NP)	96 (3.8)	92 (2.0)	95 (2.5)	95 (2.8)	94 (3.0)
NH2 (NP)	120 (3.5)	93 (2.6)	95 (3.0)	89 (4.3)	84 (2.8)
C18 (RP)	67 (4.0)	52 (2.4)	62 (2.3)	68 (3.3)	50 (3.5)
C8 (RP)	98 (5.5)	79 (2.3)	95 (3.0)	100 (3.0)	98 (4.0)
CN (RP)	70 (3.6)	52 (3.5)	59 (4.0)	62 (5.0)	60 (4.0)

[a] NP, normal-phase; RP, reversed-phase; coefficients of variation (%) in brackets (*n* = 3).

conditioning. Comparable results (75–100%) were obtained with C8 cartridge (reversed-phase), but with lowest coefficients of variation. The clean-up procedures employing Sep-Pak cartridges proved to be as efficient in the normal- (NH$_2$ and CN) as in the reversed (C8)-phase mode. Direct elution methods use a small volume of each ES and, in addition, these clean-up procedures are extremely fast, efficient and attractive because they allow a technician to prepare 10–15 samples within one working day.

This work [61] is a good example of the complexity of performing an exhaustive study of the clean-up methods for a chemical group of compounds. Since many pesticides from the same group in many cases exhibit different polarity the use of different column materials provides the best approach to study the behavior of these analytes in the different column materials. Overall, the conclusions of this work [61] were as follows and they can be applied to other types of studies:

1. The time required for clean up: lower, when Sep-Pak cartridges are used; higher for clean-up on adsorption columns e(specially for Celite/charcoal columns).
2. The facility of operation. Superior for Sep-Pak cartridges.
3. Recovery of NMC insecticides (essential parameter). Higher than 89% for alumina (ES: methylene chlorine/methanol, 99:1) and some cartridges (NH$_2$ (>84%) and CN (>92%) in normal-phase; C8 (>79%) in reversed-phase). For Celite/charcoal adsorbent columns (ES: toluene/acetonitrile, 75:25) recoveries from 77 to 105% were found; alternatively, the ES methylene chlorine/methanol (99:1) can be used for elution of the NMCs on this adsorbent (79–100% recoveries).
4. The cost of procedure. More expensive for SPE cartridges, especially if its applied to routine based analysis.

4.3 LC DETERMINATION OF PESTICIDES (EXCEPT MS DETECTION)

4.3.1 Neutral pesticides: organophosphorus, triazines, phenylureas, sulfonylureas and carbamates

The LC analysis of this group includes some thermolabile and/or polar organopho-

sphorus compounds such as dichlorvos, temephos, trichlorfon or oxydemethon-methyl. The organophosphorus pesticides of the so-called parathion group, with aromatic moieties, do not represent a problem in UV since they exhibit good chromophores. Problems arise with pesticides with poor or no chromophores, like malathion or trichlorfon. In any case, DAD detector has been routinely applied to the trace determination of many organophosphorus pesticides in water samples as shown in Refs. [67–69]. Organophosphorus pesticides are often degraded, either by hydrolysis of photolysis, to the polar transformation like phenols. In a recent paper [69] a variety of organophosphorus pesticides and their phenolic transformation products (see Fig. 4.11) were analyzed by off-line SPE followed by LC-DAD. It was found that the use of polymeric materials, like Lichrolut EN, is a better approach when a mixture of organrophosphrous pesticides together with their phenolic derivatives needs to be analyzed. Recoveries of all studied analytes are indicated in Table 4.10

Triazines can be directly analyzed by GC-NPD, and are also easily analyzed by LC-UV-DAD. Thanks to their strong absorbance at 220 nm, the detection limits obtained by the two procedures are roughly equivalent, although a lower sensitivity was reported by LC-UV in comparison to GC-NPD [70]. Many examples can be found in the literature (see [71–73] and references cited therein), and detection limits below 0.1 µg/l are frequently reported in drinking water samples after an enrichment step using both off-line and on-line procedures). The advantages of LC is that the hydroxy derivatives can be included with the
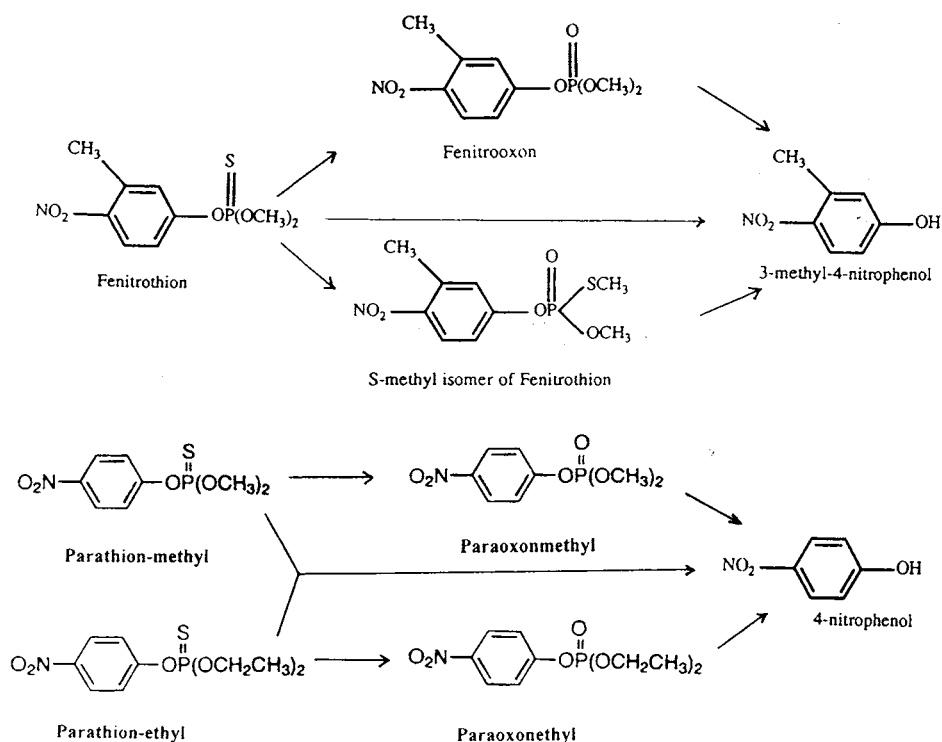
Fig. 4.11. Degradation pathway of fenitrothion, parathion-methyl and parathion-ethyl.

TABLE 4.10

MEAN PERCENTAGE RECOVERIES AND COEFFICIENTS OF VARIATION ($n = 3$) OBTAINED FOR THE PRECONCETRATION OF 100 ml OF GROUND WATER SAMPLES SPIKED AT 40 ppb WITH SOME ORGANOPHOSPHORUS PESTICIDES AND SELECTED TRANSFORMATION PRODUCTS, USING SPE FOLLOWED BY LC-DAD

Compound	Sorbent materials	
	Lichrolut EN	C18
Methyl-parathion	90 (13)	70 (10)
Fenitrothion	80 (10)	77 (11)
Ethyl-parathion	105 (9)	87 (10)
Pentachlorophenol	77 (12)	57 (10)
4-Nitrophenol	80 (13)	32 (13)
Paraoxon-methyl	82 (11)	54 (10)
3-Methyl-4-nitrophenol	84 (10)	36 (16)
Fenitroxon	79 (12)	56 (12)
Paraoxon-methyl	83 (9)	41 (13)

other transformation products [74]. The identification of the hydroxy derivatives using the UV-DAD is easy because of the characteristic of their UV spectrum. The use of on-line crossflow filtration coupled to LC permitted an effective sampling and sample clean-up of environmental water samples containing atrazines and their polar dealkylated and hydroxylated metabolites [74]. Selectivity can be increased by the use of electrochemical detectors, however a real increase in sensitivity was not noticed [75–77].

All urea herbicides and their degradation products can be determined by LC-UV. Linuron and three metabolites could be determined simultaneously [78]. After off-line or on-line enrichment steps, detection limits in drinking water are in the low 0.1 μg/l range using simply UV-DAD [79]. Phenylureas have well defined spectra with maxima around 244 nm, so that UV-DAD can give spectral confirmation. With contaminated surface water samples, a clean-up step should be applied to reach such low levels for the more polar phenylureas such as metoxuron [80].

Phenylureas are easily detected by electrochemical detection and the coupling of the ED to the UV-DAD increases the selectivity and reinforces the identification of compounds [81–84]. Nielen et al. applied LC-ED to surface water samples and found it to be a sensitive screening without any clean-up of samples at the sub μg/l level [85]. Electrode contamination could be suppressed by pulsing the electrode periodically to high potentials.

Discrimination between phenylureas and the substituted anilines, which were reported as their main degradation products, can be achieved by optimization of the LC separation. It was also performed by a combination of GC and LC techniques [86] or by an appropriate sample pre-treatment which removed the anilines [87].

Other attempts have been made to increase the sensitivity by derivatization and using a selective detector. Hydrolysis was performed on metoxuron and the product converted into a fluorescent derivative with dansyl chloride [88]. Another derivatization procedure was described by inserting a module for photodegradation of the phenylureas between the LC column and the fluorescent detector and by derivatizing the photodegradation products with *o*-phthalaldehyde [89].

The electron capture detector was used in the analysis of phenylurea herbicides after their derivatization with HFBA [90]. A packed-capillary reversed-phase liquid chromatography was coupled with ECD and showed that linuron could be determined at the sub μg/ l level in surface water [91].

One of the interesting papers recently published [16] includes the combination of in-vial solid phase extraction followed by LC-diode array. The method was applied for the trace determination of diuron and four of its metabolites in surface water and ground water. In this paper it was reported that the concentrations of some of diuron metabolites like DCPMU (N'-(3,4-dichlrophenyl)-N-methylurea) and DCPU (3,4-dichlorophenylurea) reached concentration values between 5 and 40% of the concentration determined for diuron. This indicates once more the importance of determining pesticide metabolites in pesticide monitoring programs.

Sulfonlyureas, are also becoming very popular and are replacing other herbicides, mainly because of their low application rates. Sulfonylureas are typically applied at rates less than 100 g/ha. and have low mammalian toxicity and degrade to innocuous compounds after application. In-solid phase extraction using C18 cartridges [92] for water analysis and the use of microwave assisted solvent extraction for soil samples [93] both followed by LC-UV at 226 nm have been developed. In any case, such methods were reported to be suitable for screening of sulfonylureas in environmental samples but both methods indicated that LC-MS would be required for definitive confirmation.

The LC analysis of carbamate pesticides is preferred because of their thermal degradation. However, not all the compounds can be detected by UV. Those containing a UV chromophore, such as carbofuran, carbaryl, benomyl, carbendazim, can be directly detected by UV or UV-DAD after an off-line or on-line enrichment step [61,79,94].

Other carbamate pesticides could be directly determined using electrochemical [95–98] or fluorescence detectors [99,100]. A high increase in sensitivity was obtained using fluorescence. The electrochemical detection was reported for eight carbamates using a flow-cell with a wax-impregnated graphite electrode working at positive potentials [96]. Detection limits were below 5 ng. Andersen et al. [97,98] used kel-Fl-graphite electrode for the direct determination of carbamates in water because they were able to obtain detection limits in the picogram range. In order to avoid the accumulation of reaction products at the surface of the electrode, they applied an in situ cleaning by pulsing the electrode periodically to extreme potentials. Using a platinum working electrode, another study reported detection limits in the nanogram to picogram range depending on the compound and on the potential applied [101].

The number of carbamates with direct UV, electroactive or fluorescence properties is limited and for the other carbamates, precolumn or postcolumn derivatization is needed. Derivatization methods applied to carbamates have been recently reviewed [102,103]. A precolumn mode was described by Lawrence and Leduc [104]. The carbamates were hydrolyzed in phenols, which could react with dansyl chloride to produce dansyl derivatives which were then separated and detected. The postcolumn mode was introduced by Moye et al. [105] and has been widely recognized for its sensitivity and selectivity, so an automated on-line derivatization device is commercially available, and it is the basis of the official EPA Method 531.1 and EPA Method 8318. The N-methylcarbamates are separated on a C18 silica column and are also hydrolyzed in methylamine and phenols; then methylamine is derivatized with OPA or with a mixture

of OPA with 2-mercaptoethanol (OPA/MERC) in order to produce highly fluorescent 1-hydroxyethylthio-2-methylisoindole

The method was improved and provided detection limits of 0.1–0.5 ng [106]. These detection limits can be routinely obtained with Pickering postcolumn derivatization instruments. This method is very selective since only *N*-methylcarbamates and *N*-methyl carbamoyloximes are then detected.

The use of a solid phase reactor containing an anion exchanger for the hydrolysis of the *N*-methyl carbamates simplified the postcolumn derivatization system [107,108]. Postcolumn solid phase hydrolysis was applied to the analysis of a large number of *N*-methyl carbamates and their sulphone and sulphoxide degradation products, using an off-line solid phase extraction from 50-ml ampoules with detection of compounds at levels below 30 ng/l [109]. The procedure was automated with an on-line preconcentration with same detection levels from 5-ml samples and the chromatogram corresponding to the analysis of a 5-ml surface water sample [110].

A postreaction detector was also developed for the determination of the fungicides thiram and disulfiram [111]. Detection was based on a postcolumn complexation of these analytes on a solid state reactor packed with finely divided metallic copper to form a colored copper complex, copper(II) *N,N*-dimethyldithiocarbamate, with an absorption maximum at 435 nm.

4.3.2 Acidic pesticides

LC was used in order to avoid the necessary derivatization procedure in GC. The simplest method is based on an enrichment step, a reversed-phase separation and direct UV-DAD detection, since phenoxyalkanoic acids have characteristic UV spectra with two maxima, a first one at 230–235 nm and a second one, less intensive, at 280 nm [112,113]. The second wavelength was selected because fewer interfering compounds are detected at 280 nm in comparison to 230 nm [114]. LC determination of phenols and derivatives, which are the main degradation products have been recently reviewed [115]. Chlorophenols can be detected at 220 or 280 nm in the same way as phenoxyalkanoic acids.

In order to avoid the number of interferences detected by UV, some more selective detection modes were investigated. Simple indirect photometric detection at 510 nm was described, with an addition of 1,10-phenanthroline in the mobile phase [116]. Electrochemical detection was also tested for the detection of chlorophenols [117,118]. The selectivity was effectively increased with a lower background, but the sensitivity was not better than that obtained with UV. For lower chlorophenols, a postcolumn photo conversion to phenol by UV irradiation followed by fluorescence detection was described and was found to be very selective when applied to surface water samples [119]. Precolumn derivatization was also described to enhance the selectivity and/or sensitivity for the determination of phenol and chlorophenols [120,121].For phenoxyalkanoic acids, selectivity is usually provided by the sample handling step, taking into account that these compounds can be ionized [113,122,123].

There are some of the acidic pesticides that need a specific method of analysis. This is the case for dimethyl tetrachloroterephthalate (DCPA) and its metabolites like monomethyl tetrachlroterephthalate (MM) and tetrachrloroterephthalic acid (TCPA) which are currently determined by US EPA Method 515, which employs GC-with electron

capture detection, including a very labor intensive steps, with manual extraction of large volumes of organic solvents, vaporization/concentration and derivatization [1]. Recently [124] a method was developed involving direct injection with a sample loop of 2 ml into an HPLC systems incorporating ion pair and UV-DAD detection. This method allowed detection limits at low ppb range and includes also an additional confirmation of the positive analytes via liquid–liquid extraction and dertivatization followed by GC-MS.

4.3.3 Very polar/ionic pesticides: glyphosate, diquat and paraquat, aminotriazole

Glyphosate is a very broad spectrum non-selective post-emergence herbicide which widely used. Its trace determination, as well as that of its metabolite aminomethyl phosphonic acid (AMPA) is difficult because it is highly soluble in water, and insoluble in organic solvents. Although the GC analysis of glyphosate has been described through derivatization with the reagent *N*-methyl-*N*(tert-butyldimethylsilyl)trifluoroacetamide in dimethylformamide [125], or through derivatization with pentafluoropropionic anhydride plus trifluoroethanol [126], the characteristics of glyphosate and AMPA make them more easily analyzed by LC. Since the molecules do not posses any chromophore, they need to be derivatized for detection. Both pre- and postcolumn derivatization have been developed. Derivatization in precolumn procedures is carried out with 9-fluorenyl-methyloxycarbonyl chloroformate (FMOC-Cl) yielding highly fluorescent derivatives. This method was recently applied by Sancho et al. [127] to soil samples. After extraction in alkaline media, neutralization and derivatization, two different LC methods have been applied. The first one is focused on the determination of glyphosate only and require a liquid–liquid extraction step with ethyl acetate for the removal of the reagent excess prior to RPLC analysis with a C18 analytical column under isocratic elution conditions. The second makes use of coupled-column LC for on-line sample clean-up of diluted extracts and allows the determination of both glyphosate and AMPA. Other pre-derivatization agents such as 1-flouro-2,4-dinitrobenzene [128] and *p*-toluenesulfonyl chloride [129] have been used for detection by UV-Vis, but with lower sensitivity.

Postcolumn procedures use derivatization with OPA-MERC, and fluorescence detection [130–132]. Most of the procedures utilized the commercial postcolumn reactor developed for the analysis of carbamates, but the procedure is slightly different: glyphosate is oxidized with calcium hydrochorite to glycine which is then coupled to OPA-MERC to give a fluorophore. The US EPA method consists of a direct injection of the aqueous samples with a limit of detection of 6 μg/l in drinking water. Other methods commonly reported combine an enrichment step in order to obtain lower LODs. The extraction of glyphosate from aqueous matrices is difficult owing to its high solubility in water and requires the use of anion exchangers. The separation of glyphosate and AMPA is easily performed using a cation exchange column. Recently an improvement of EPA methods for glyphosate analysis was published [133]. The quantitative trace determination of glyphosate and its major metabolite, aminophosphonic acid (AMPA) in natural water was achieved by means of ion exchange chromatography. Fifty milliliters of a natural water sample were preconcentrated by a two-step procedure: first the sample was percolated through a polymeric cartridge, LiChrolut EN, then through an anion exchange column mechanism, and finally analyzed by ion exchange chromatography followed by postcolumn reaction and coupled to a fluorimetric detector. Linear calibration graphs were

obtained between 5 and 200 μg/l. Limits of detection ranged from 2 μg/l for glyphosate and 4 μg/l of AMPA. Figs. 4.12–4.14 show the scheme of the system, the chemical reaction and a chromatogram of the trace analysis of glyphosate and AMPA in a natural water sample, respectively.

Diquat and paraquat are widely applied and are difficult to GC analyze and their analysis is usually performed by LC or, more recently, by CZE. The LC method is described in US EPA Method 549.1. Diquat and paraquat are quaternary amines that can be easily separated using ion exchange LC or ion-pairing LC, and easily detectable by UV-DAD. Because of their characteristic UV spectra, they cannot be analyzed at the same wavelength; paraquat is detected at 257 nm and diquat at 308 nm. [1]. With an off-line trace-enrichment step from 250 ml of water sample, a final extract of 250 ml and an injection volume of 10 ml, the minimum detectable concentration is in the range 0.4–0.5 μg/l. The comparison between US EPA Method 549.1 and the LC-electrospray ionization-mass spectrometry (ESI-MS) method has been published [134]. The method involved the preconcentration on ENVI-8 DSK SPE disks. No breakthrough was observed up to 500 ml of different types of waters, like tap water, lake water, river water and ground water. The LC-ESI-MS isotope dilution method correlated well width the EPA method involving LC-UV.

Aminotriazole is widely used for agricultural and industrial usage. Its low volatility, high polarity and high solubility in water (280 g/l) make it impossible to analyze by gas chromatography, and very difficult to extract from water, although GC analysis after acetylation was reported. A review was recently published summarizing all the possibilities for both its extraction and its detection after UV [135]. Aminotriazole is too polar to be analyzed by reversed-phase chromatography, where elution with pure water as a mobile phase occurs close to the void volume retention time. Normal phase chromatography is not the best technique, due to the impossibility of injecting a high amount of aqueous solvents
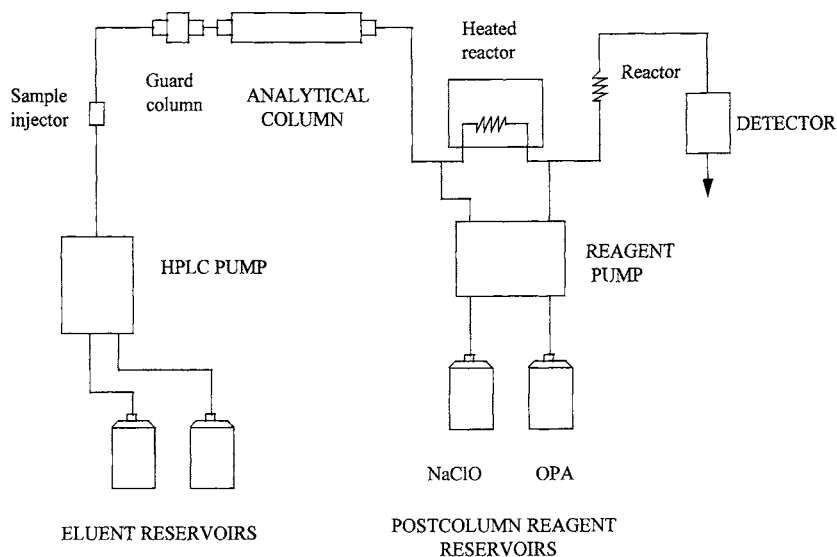
Fig. 4.12. Instrumental configuration for the analysis of glyphosate by post-column reaction.

Oxidation step:

$$HOOC-CH_2-NH-CH_2-\overset{\overset{O}{\|}}{\underset{\overset{|}{OH}}{P}}-OH \xrightarrow[36\ ^{\circ}C]{OCl^-} HOOC-CH_2-NH_2$$

Glyphosate   Glycine

Derivatization:

$$R-CH_2-NH_2 + \overset{CHO}{\underset{CHO}{\bigcirc}} + (CH_3)_2NCH_2CH_2SH \longrightarrow \overset{SCH_2CH_2N(CH_3)_2}{\bigcirc NCH_2R}$$

R = HOOC   Glycine

R = H$_2$O$_3$P   AMPA
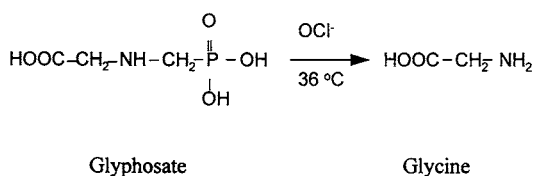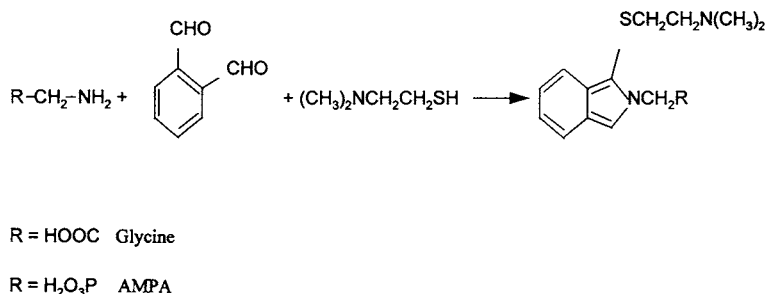
Fig. 4.13. Post-column derivatization step for the analysis of glyphosate in water samples.

in the columns used in normal-phase mode and to the low solubility of aminotriazole in organic solvents. But, since it can be ionized at low pH, ion-pair chromatography or exchange chromatography are the appropriate methods. The detection by UV is not sensitive at all owing to the lack of chromophore, even when derivatization techniques are made. The most sensitive detection is electrochemistry. With available concentration techniques, no robust methods can detect routinely aminotriazole below the 0.3–0.5 μg/l level in drinking water.

4.3.4 US EPA methods

The US EPA methods using LC are listed in Table 4.11. These methods complement the GC methods to cover almost any pesticide and transformation products included in the EPA list. These methods include multiresidue analysis, for chlorinated pesticides, and are specific for very polar/ionic pesticides, such as glyphosate, diquat and paraquat. Method 531, which uses postcolumn reaction with fluorescence detection provides good detection limits by direct injection of 200 μl of an aqueous sample, showing thus the high sensitivity of this detection mode. A modification of this method for the analysis of carbamate pesticides in waste water matrices, that includes extraction of carbamate pesticides form water and/or soil samples is also reported in Method 8318. An on-line SPE method was introduced by the US EPA for the determination of chlorinated phenoxyacids in water matrices and it is also indicated in Table 4.11. This represents a progress in the US EPA methods and, in a way, a closer approach to European monitoring programs, like the Rhine Basin, where automated on-line SPE techniques are currently applied. Three methods
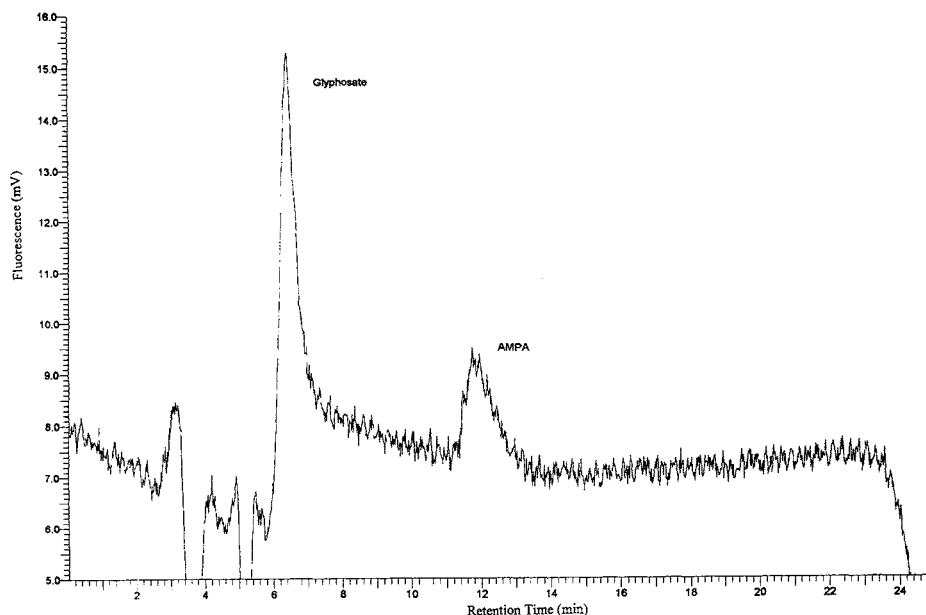
Fig. 4.14. Chromatogram corresponding to the extraction of 50 ml of a lake water sample and analysis by ion exchange chromatography with postcolumn reaction coupled to a fluorimetric detector. The sample contains glyphosate at 15 ppb and AMPA at 6 ppb. For extraction and chromatographic conditions see text.

based on the use of LC-MS, methods 553, 8321A and 8325 are also reported in Table 4.11. These methods are, at present, not very appropriate, since they are based on the use of either thermospray or particle beam interfaces in LC-MS. We will discuss in a latter part of this chapter the fast growing LC-MS interfacing systems, like atmospheric pressure ionization interfaces, such as APCI or electrospray (ESI). These new interfaces permit one to achieve better LODs than the old type PB or TSP, but the methods are reported here because they were then first EPA methods using LC-MS as a methods for determining different groups of pesticides. Although the detection part can be improved by using the current API interfaces, still the sample preparation step is valid and it can be used in combination with these API interfaces.

4.3.5 Multiresidue methods

The potential of LC for multiresidue analysis was shown by Di Corcia and Marchetti [112]. In one run, they achieved the separation of 71 neutral and basic pesticides using a 250 × 4.6 mm ID C18 column and a linear water–acetonitrile gradient. The pesticide mixture is re-analyzed using a cyano-propyl modified silica column as confirmatory column. The term multiresidue analysis can be really applied to this separation, since the standard mixture contain pesticides used as insecticides, herbicides, fungicides, nematicides and acaricides, representative of all the common groups, i.e. carbamates, phenylureas, triazines, triazinone, organophosphorus, acetanilides, dinitroanilines and even one

TABLE 4.11

SUMMARY OF THE US EPA METHODS USING LC TECHNIQUES

EPA Method 531.1: Determination *N*-methylcarbamoxyloximes and *N*-methylcarbamates in ground water by direct-aqueous-injection-LC, postcolumn derivatization and fluorescence detection

Direct injection of water samples

LC column: C18 silica column

After elution, hydrolysis with 0.05 M NaOH at 95°C, reaction with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol to form a highly fluorescent derivative

Detection with fluorescence

Compounds detected (LOD in the range 0.5–4.0 μg/l): aldicarb, aldicarb sulfone, aldicarb sulfoxide, propoxur, carbaryl, carbofuran, 3-hydrocarbofuran, methiocarb, methomyl, oxamyl

EPA Method 555: Determination of chlorinated acids in water by LC with UV-DAD

Liquid–solid phase extraction (20–100 ml); esters are included after hydrolysis with 6 N NaOH followed by acidification with H_3PO_4

LC column: C18 silica column, 250 × 4.6 mm ID; primary column: ODS-AQ, 5 mm; (YMC Ltd.) and confirmatory column; 300 × 4.6 mm ID, Nova-Pak C18 (Waters)

Mobile phase: water (H_3PO_4, 0.025 M) – acetonitrile gradient (10- 90% acetonitrile) in 30 min

UV-DAD detection

Compounds detected (LOD in the range 0.1–0.5 μg/l with extraction of 100 ml): acifluoren, bentazone, chloramben, 2,4-D, 2,4-DB, dicamba, 3,5-dichlorobenzoic acid, dichlorprop, dinoseb, 5-hydroxycamba, MCPA, MCPP, 4-nitrophenol, pentachlorophenol, picloram, 2,4,5-T, 2,4,5-TP

EPA Method 547: Determination of glyphosate in drinking water by direct-aqueous-injection-LC, postcolumn derivatization and fluorescence detection

Direct injection of filtered water samples (200 ml)

LC column: cation exchange LC column, 250 × 4.6 mm, Bio-Rad Aminex A-9, K^+ form at 65°C; guard column: C18 packing

Mobile phase: isocratic, 0.005 KH_2PO_4 and methanol, 96:4, v/v

After elution at 65°C, oxidation with calcium hypochlorite. The product (glycine) is coupled with *o*-phthaldehyde (OPA) and 2-mercaptoethanol at 38 °C to give a highly fluorescent derivative

Detection with fluorescence, excitation at 340 nm and detection of emission measured at >455 nm

Detection limits: 6.0 μg/l in reagent water, 9.0 in ground water.

EPA Method 549.1: Determination of diquat and paraquat in drinking water by liquid-solid extraction and LC with UV detection

Solid-phase extraction from 250 ml of sample, adjusted to pH 10.5 using a C8 cartridge or disk prepared for the reversed-phase, ion-pair mode

LC column: Hamilton PRP-1, 5 mm, 150 × 4.1 mm at 35.0 °C

Mobile phase: isocratic, ion-pair mobile phase

UV detection at 308 nm (diquat) and 257 nm (paraquat) or UV-DAD

Detection limits: 0.4 μg/l for diquat and 0.8 μg/l for paraquat

EPA method 553: Determination of benzidines and nitrogen-containing pesticides in water by liquid–liquid extraction or liquid–solid extraction and reverse phase high performance liquid chromatography-particle beam mass spectrometry

1 l water samples extracted by LLE with methylene chloride or by C18 or neutral polystyrene–divinylbenzne polymer, either in cartridge or disk format

LC column: 15–25 cm × 2 mm stainless steel Waters C18 or equivalent packed with 4–10 μm particles

Detection limits (1–30 ppb) for benzidine, benzoylprop ethyl, carbaryl, 3,3'-dichlorobenzidine, 3,3'-diemthoxybenzidine, 3,3'-dimetylbenzidine, diuorn, linuron, monuron, rotenone and siduron. The method indicates that other analytes like aldicarb sulfone, carbofuran, methiocarb, methomyl, mexacarbate and *N*(1-naphthyl) thiourea may be analyzed by this method but no data are reported

TABLE 4.11 (*continued*)

EPA Method 8318: Determination of *N*-methylcarbamates by high performance liquid chromatography
  The method is used to determine the concentration of *N*-methylcarbamates in soil, water and waste matrices
  Water matrices (domestic waste water, aqueous industrial wastes and leachates): 100 ml of water are
  extracted with methylene chloride
  Soils and wastes: 20 g shaking with acetonitrile for 2 h
  Clean-up with C18 cartridges, filtered and eluted in a C18 analytical column
Final determination as the US EPA method 531.1
  Due to the specific nature of this analysis, confirmation by a secondary method is not essential
  Sensitivity: depends very much on the level of interferences from the complex matrices analyzed rather than
  on the instrumental conditions
  Compounds detected (LOD in the range 1.7–9.4 μg/l, for water and from 12–50 μg/kg for soils): aldicarb,
  aldicarb sulfone, carbaryl, carbofuran, dioxacarb, 3-hydrocarbofuran, methiocarb, methomyl, oxamyl,
  pormecarb and propoxur

EPA Method 8321 A: Solvent extractable non-volatile compounds by HPLC/thermospray/mass spectrometry or
UV detection
  This method covers a wide range of organic pollutants like disperse azo dyes, organophosphorus pesticides,
  chlorinated phenoxy acid compounds and carbamates in waste water, ground water and soil/sediment matrices
  Extraction, from soil samples, with microextraction, only 1 g of samples is needed
  Normal extraction, 50 g using acetone–diethyl ether (20:80) and from water matrices, 1 l using diethyl ether
  as solvent
  LC conditions in reverse phase using positive ionization mode of the thermospray MS
  Compounds detected (LOD in the range of 0.4–4 ppb, for water and from 0.01 to 0.1 μg/g for soil) aldicarb
  sulfoxide, alficarb sulfone, oxamyl, methomyl, 3-hydroxycarbofuran, fenuron, benomyl/carbendazim,
  aldicarb, aminocarb, carbofuran, propoxur, monuron, bromacil, tebuthiuron, carbaryl, fluometruon, propham,
  propachlor, diuron, siduron, methiocarb, barban, linuron, chlorpropham, mexacarbate, chloroxuron, neburon,
  dalapon, dicamba, 2,4,-D, MCPA, dichlorprop, MCPP, 2,4,5-T, 2,4,5-TP (Silvex), 2,4DB and Dinsoeb
  Other analytes reported with this method but with indication of recovery data but not of limits of detection,
  are: dimethoate, dichlorvos, naled, fensulfotion, methyl parathion, phorate, disulfoton and merphos

EPA Method 8325: Solvent extractable compounds by HPLC-particle beam MS
  This method involves the same compounds and final analytical determination as Method 553, but the method
  is now applied to waste water samples
  1 l water samples extracted by LLE with methylene chloride or by C18 or neutral polystyrene–divinylbenzne
  polymer, either in a cartridge or disk format
  Detection limits (2–25 ppb) for benzidine, benzoylprop ethyl, carbaryl, 3,3′-dichlorobenzidine, 3,3′-
  diemthoxybenzidine, 3,3′-dimetylbenzidien, diuron, linuron, monuron, rotenone and siduron. The method
  indicates that other analytes like aldicarb sulfone, carbofuran, methiocarb, methomyl, mexacarbate and
  N(1-naphthyl) thiourea may be analyzed by this method but no data are reported

organochlorine and one pyrethroid. In the same paper, the separation of 18 various acidic pesticides is also represented using the same columns as for the base-neutral mixture, but with an acidic mobile phase.

Multi-residue analysis is valuable provided all the compounds are extracted in one run together. This is the case of the work of Di Corcia. However, in other cases the authors will still use the term multiresidue analysis but two extraction protocols will be used. This is the case when very polar and very apolar compounds are present. Pichon et al. [136] used a C18 silica-based disk and divinylbenzene disk for a wide range of pesticides, with a log K_{ow} varying from 1 to 6. The purpose of this method was to analyze all the pesticides

incorporated in the recently published French priority list of pesticides. This list contains 20 pesticides, from very apolar ones like endosulfan to very polar ones like aminotriazole and oxydemeton methyl.

On-line SPE and LC are particularly well adapted to multiresidue analysis. From a 150-ml sample, LODs are lower than 0.1 μg/l in drinking water and in the range 0.1–0.5 μg/l in surface water. Liska et al. presented a rapid screening on-line SPE-LC method designed for an early-warning system of over 50 pesticides in surface water [137]. It allowed the separation of almost all the compounds with LODs of about 1–5 μg/l after a preconcentration of 30 ml of water. The multriresidue analysis of 12 pesticides used in rice cultures was recently reported by automated on-line SPE-LC-DAD [138]. In this example the influence of humic substances present in estuarine waters containing the rice pesticides were studied. Levels of humics varied from 5 up to 80 mg/l, that is the worst case in estuaries of tropical regions. The effect of the pH on the on-line SPE extraction of rice pesticides spiked in estuarine water containing 10 mg/l of humic substance is shown in Fig. 4.15. It can be noticed that using neutral pH is the best situation for a complete extraction of all pesticides without any serious interference from the water matrix.

Multiresidue methods have been developed using LC with postcolumn reaction detection. Over 100 analytes from the EPA's National Survey of Pesticides in Drinking Water Wells were screened for response using postcolumn photolysis followed by fluorescence, electrochemical analysis of conductivity detection [139]. LC-photolysis with electrochemical and fluorescence detection were shown to be suitable and complementary. The first combination responds to several sulfur-containing pesticides whereas the combination photolysis-FD responds to many nitrogenous pesticides. The conductivity detection was not suitable for sensitive multiresidue determination. Approximately half of the compounds from the list could be determined in low nanogram amounts using the two detection systems and multiseparation with gradient reversed-phase LC.

4.4 LC-MS

The on-line combination of liquid chromatography (LC) and mass spectrometry (MS) it is a very convenient and robust technique for the analysis of polar pollutants. Five books and book chapters [140–147] were published in the last few years, providing excellent overviews of fundamental aspects and environmental applications of LC-MS.

The main problem to overcome in LC-MS is the transfer of analyte from the liquid phase into a high vacuum gaseous-phase. To accomplish the analyte transfer, different interfacing techniques have been developed over the past decade. The nowadays often applied and commercially available interfaces can be divided into two major groups; the nebulization interfaces and the analyte-enrichment interfaces [146]. In the former group, the eluent is nebulized by means of heat (thermospray, TSP), pressure or the application of an electric field (electrospray ionization, ESI) at the tip of the spray capillary. Also combinations of nebulization techniques have been developed (pneumatically assisted electrospray and the heated pneumatic nebulizer). In general, within this group, we will give examples of pesticide analysis using four different types of interfaces: TSP, ESI, high-flow pneumatically assisted electrospray or ionspray (ISI) and APCI.

In the analyte-enrichment interfaces, the analyte is separated from the solvent flow after
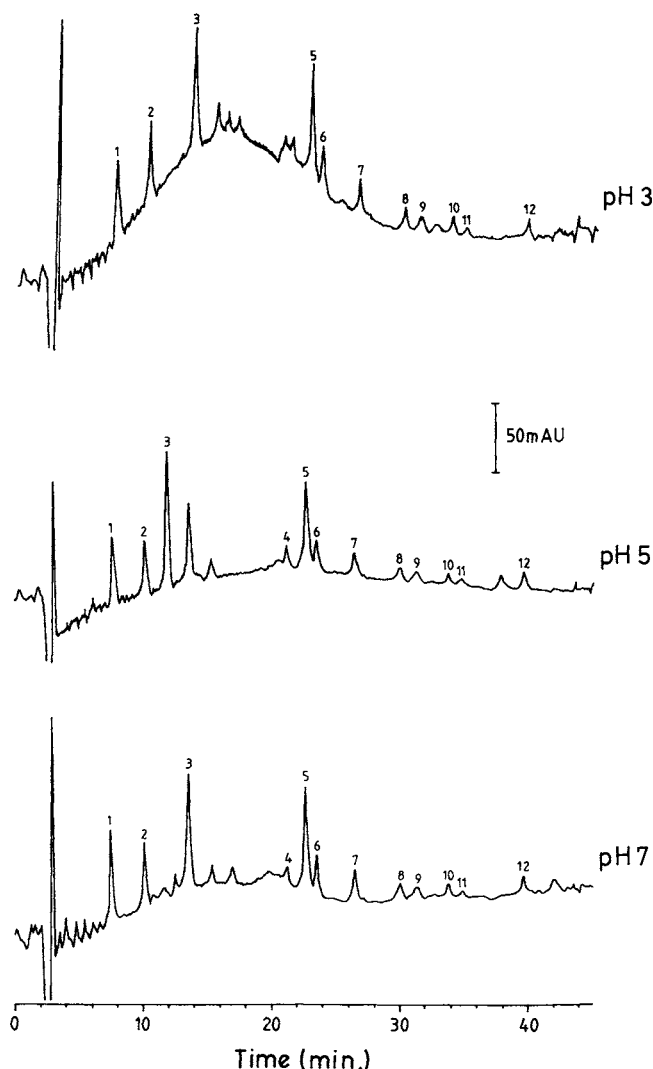
Fig. 4.15. Effect of the co-extraction of humic acids (10 mg/l) at different pH on the preconcentration of 75 ml of estuarine water spiked at 2 ppb of each pesticide. Peak number: (1) carbendazim, (2) deisopropylatrazine, (3) deethylatrazine, (4) carbofuran, (5) atrazine, (6) diuron, (7) propanil, (8) molinate, (9) alachlor, (10) parathion-ethyl, (11) diazinon and (12) trifluralin.

nebulization using a beam separator (particle beam, PB) or the subsequent evaporation of the eluent in different vacuum regions (moving belt). An excellent review on the use of PB was published in the last few years [148].

During the last few years, PB and TSP interfacing system have been practically totally replaced by ESI and APCI. For this reason, and considering that the book published recently [1] reports quite a lot of information on these interfaces as well other books [144–157], and because a specific chapter of this book is devoted to LC-MS, it was

decided to delete the application on PB and TSP to pesticide analysis. However, due to their importance as legal methods, the EPA methods involving TSP and PB will be briefly discussed.

4.4.1 EPA methods

Of the different nebulizer type of interface methodologies, TSP and PB interfacing system were widely used in environmental analysis. In this respect the US EPA has published two methods involving PB [149,150] and one involving TSP [151].A comparison between the US EPA Method 515.1, for chlorinated pesticides in water involving derivatization and GC-ECD determinations, and LC-PB-MS was carried out [152]. Table 4.11 summarizes these LC-MS EPA methods.

Method 553 involves extraction of 1 l of water by using dichloromethane LLE or SPE followed by LC-PB-MS [149]. The pesticides determined are listed in Table 4.11 with LODs varying between 4 and 31 ppb. Similarly, US EPA Method 8325, uses PB-MS and is applicable to the same list of compounds. This method is just an extension of the previous method for more complex matrices. In this method it is indicated that the LODs are compound and matrix dependent. This is known in environmental analysis, and specially using LC-MS techniques. In the case of complex waste water matrices, the LODs will be poor compared to clean drinking water matrices. LC-PB-MS has been implemented by EPA especially for its possibility of identifying unknown analytes in complex waste waters due to the possibilities of EI spectra.

The comparison of US EPA Method 515.1-using GC-ECD with prederivatization- and LC-PB-MS for the determination of various acidic chlorinated acids in water reported LODs varying from 0.7 up to 7 ppb when using LC-PB-MS whereas using EPA Method 515.1 the LODs were always below 1 ppb, with approximately one order of magnitude better sensitivity. An advantage of LC-MS over GC-ECD was noticed in the determination of 4-nitrophenol and 3,5 dichlorobenzoic acid, that offered derivatization problems under the current EPA method. For this reason, the LC-PB-MS method was recommend for all the groups of analytes

Another US EPA method involving LC-MS incorporates a TSP interface [155]. In that case, the method is applicable to the determination of a wide range of analytes, including dyes, organophopshorus pesticides, chlorinated acids and carbamates in waste water, ground water and soil/sediment matrices. This is the US EPA LC-MS method that incorporates the longest list of analytes to be determined. In that specific method, calibration is carried out by using either polyethylene glycol (PEG) 400 or 600. The method was widely applied to the determination of various pesticides like organophosphorus, carbamates and acidic pesticides, although problems associated with thermally labile pesticides like trichlorfon were detected when using such types of interfaces. The LODs, after preconcentration of a 1-l water sample, generally varied between 0.2 and 5 ppb and it is more universal than LC-PB-MS although the structural information obtained by LC-TSP-MS is poor compared to PB. The problem with the proposed method is that it includes the analysis of thermally labile pesticides like trichlorfon, methiocarb and a few others that can easily decompose under TSP interface temperatures. For this reason, before applying this proposed method, it is important to know which are the target pesticides to be determined. The method can be easily applied to chlorinated acids.

4.4.2 LC-ESI-MS

In this section we will discuss two LC-MS coupling techniques; ESI and high-flow pneumatically assisted electrospray or ionspray (ISI). The nebulization in the ESP and ISI interfaces does not use heat and thus no thermally assisted degradation is expected. The major differences for routine pesticide analysis between these two interfaces is that in the case of conventional ESI, only 20–50 ml/min are directed to the MS source, being neces- sary to split the LC flow rate whereas in the case of ISI, up to 1 ml/min can be handled by the ISI interfaces, with no need of splitting the LC eluent. Several applications in pesticide analysis have been reported in the literature in the last few years [153–177]. However, due to the recent advances in interfacing systems, nowadays almost all the manufacturers offer interfacing systems with high flow rates and they are still called ESI. For this reason we will not use the term ISI and only ESI will be used, either for low or high flow rates in ESI.

Ion formation in ESI is due to the evaporation of ions from charged droplets generating rather cold ions with nearly no excess in internal energy and thus little or no fragmentation is observed. For the enhancement of the sampling efficiency of the ions formed an extrac- tion potential is applied, focusing the ion spray towards the entrance orifice. Increasing this voltage will increase the total ion current. Moreover, higher voltages have been proved to induce fragmentation of various type of analytes, comparable to fragmentation observed in collisionally activated dissociation (CID) mass spectra [155].

One of the most commented aspects of LC-ESI-MS is the formation of the $[M + Na]^+$ ion. The sodium ions present originate as impurity in the methanol solution. Since under ESI the vaporization takes place from the solution, it was reported that 90% of the observed ions are due to ions present in the solution. The second point arises from the use of methanol or acetonitrile in the mobile phase. Acetonitrile certainly decreases the abundance of the sodium-cationized ions vs. methanol as reported for various organopho- sphorus pesticides, monuron and carbofuran [158]. The sodium ion addition is thermo- dynamically favored and occurs preferentially in dilute solution, but in more concentrated solution the sodium ions are depleted and protonation becomes dominant. In general we can indicate that the formation of abundant adduct ions with sodium is related; (i) to the use of methanol in the mobile phase, (ii) to the fact that they are compound dependent and showed very low abundances of the sodium adduct ion for triazine herbicides whereas for phenylurea the sodium adduct ion was the base peak [157] and (iii) to the fact that they are dependent on the concentration of the compound and on the cone voltage used. In this respect it was noticed that the $[M + Na]^+$ ions can exhibit high or low abundances versus the increased cone voltage. In this respect, the $[M + Na]^+$ ion was stable for phenylurea herbicides, being impossible to obtain any fragmentation even at a cone voltage of 100 V. Compared with triazine herbicides this behavior was totally different and it was attributed to the stability of the aromatic structure of the phenylurea herbicides with the sodium adduct ion, compared to triazines [157]. Again, the $[M + Na]^+$ ion is stable under different extraction voltages and is compound dependent. In addition, if additives such as NH_4^+, H^+, are added to the eluent, then the signal intensity of the sodium adduct ions diminishes sharply.

The formation of adduct ions others than $[M + H]^+$ is not very favorable, because the variations of the salt content in the organic solvent can reflect the variations of the abundances of both fragment and parent ions. Addition of an acidifying agent into the

mobile phase is not enough to suppress the $[M + Na]^+$ ion, so an elegant solution was proposed by Di Corcia et al., that used a prior distillation step for eliminating sodium and other cations from the organic solvent [163].

The major attraction of LC-ESI-MS applied to pesticide analysis is the low detection limits of this technique for determining pesticides in water when combined with SPE. LODs for organophosphorus pesticides varied from 0.01 to 0.2 ppb using only 200–300 ml of water after off-line SPE using disks or cartridges [156,158]. An example on the analysis of various thermally labile carbamates showed that it was possible to carry out direct analysis of various carbamates at the 0.1 ppb level in water by large volume injection of 0.5 ml of water [160].

Many examples are reported in the literature of the use of LC-ESI-MS for pesticide analysis. Among the different groups and examples studied we should mention applications for trace determination of organophosphorus [156,158], carbamates [160], triazines [157,164,170], pehnoxyacids [161,162,172,173], imidazolinone [174,185], glyphosate [176,177] and multiresidue methods involving different pesticide groups [163,172]. As an example of the application of LC-ESI-MS to environmental analysis, Fig. 4.16 shows a chromatogram obtained by time scheduled SIM of a drinking water extract spiked with a mixture of base/neutral and acidic pesticides at 25 ng/l [172]. Using this system, LODs were generally below 2 ng/l when preconcentrating 2 l of drinking water samples with 0.5 g of graphitized carbon black.

Typical diagnostic ions were generally obtained when increasing the cone or fragmentor voltage. This a very important feature of ESI since it is a technique that can be used for identification purposes of unknown pesticides, contrary to most of the TS applications that usually give very poor structural information and consequently it is used for confirmation purposes of target analytes.

The confirmation of unknown pesticide metabolites is a very important issue in environmental pesticide analysis. Several papers [166–169] have dealt with the identification of polar pesticide metabolites of chloracetanilide herbicides and especially alachlor. The first LC-ESI-MS identification of ionic pesticide metabolites of chloroacetanilide herbicides was reported recently [166]. The mass spectra and fragmentation ions for acetochlor oxanilic acid, alachlor oxanilic acid and metolachlor oxanilic acid are reported in 12. The base peak at 146 for acetochlor oxanilic acid is formed via breaking of the carbon–nitrogen bond between the aromatic ring and the amide nitrogen. A proton is transferred from the alkyl group of the aromatic ring to the nitrogen which forms the 146 ion. The 146 base peak ion occurs at a cone voltage of 40 V. However, at a cone voltage of 20 V, the base peak ion is the molecular ion. Therefore, a cone voltage of 40 V was chosen for sample analysis in order to distinguish acetochlor oxanilic acid from alachlor oxanilic acid, which both have the same molecular ion $[M–H]^-$ at 264. For alachlor oxanilic acid the base peak ion was at m/z 160. This ion is formed by the transfer of either of the ethyl groups from the aromatic ring to the amide nitrogen. For metolachlor oxanilic acid, the fragmentation occurs between the alkyl-side chain and the amide nitrogen, which gives the base peak of mass 206.The negative ion spectra in electrospray for the three sulfonic acids metabolites gave only the molecular ion (Table 4.12). Thus, acetochlor and alachlor sulfonic acids have the same molecular ion at 314. Metolachlor sulfonic acid has its molecular ion at 328. These spectra were obtained at 20 and 40 V. Nevertheless, minor fragmentation was seen for both acetochlor and alachlor sulfonic acids at a cone voltage of
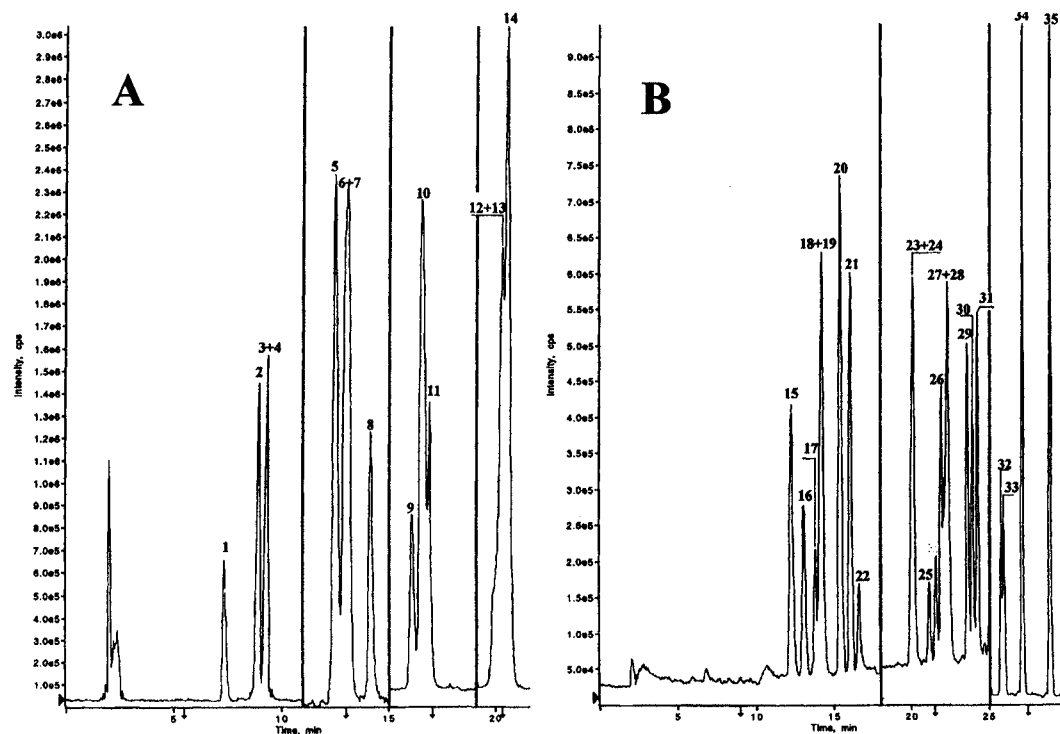
Fig. 4.16. Time scheduled SIM chromatogram obtained under LC-ESI-MS for (A) neutral and (B) acid fraction obtained injecting 20:200 of a final extract relative to 2 l of drinking water spiked with the herbicides at levels of 25 ng/l each. Peak number: (1) cyanazine, (2) imazamethabenz methyl, (3) simazine, (4) metribuzine, (50 atrazine, (6) isoproturon, (7) diuron, (8) propazine, (9) linuron, (10) ametryne, (11) terbutylazine, (12) alachlor, (13) metolachlor, (14) terbutryn, (15) imazethapyr, (16) triasulfuron, (17) imazaquin, (18) dicamba, (19) metsulfuron, (20) bantazone, (21) chlorsulfuron, (22) rimsulfuron, (23) bropmoxynil, (24) tribenuron, (25) 2,4-D, (26) MCPA, (27) ionxynil, (28) bnesulfuron, (29) fluazifop, (30) dichlorprop, (31) mecoprop, (32) 2,4-D, (33) MCPB, (34) haloxyfop and (35) diclofop.

60 V. Unfortunately, their sensitivity is low at this cone voltage and it is not suitable for routine analysis. Therefore, chromatographic separation is required for identification of acetochlor and alachlor sulfonic acids or the use of tandem MS. This was proposed in another article [167] that permitted the determination of all sulfonic degradates of chloro-acetanilide herbicides by LC tandem MS with ESI.

In summary we can indicate that LC-ESI-MS is a very useful technique and in a way, a routine method, for analyzing pesticides in water, after SPE. This combination gives enough structural information, when increasing the extraction voltage of the system, that permits the identification of unknown analytes. But the most relevant future is the sensitivity that permits, by handling less than 1 l of water samples (from 200 to 500 ml), one to reach LODs below 0.1 ppb and so it can be used for compliance with EU directives for drinking water. LC-ESI-MS can be used for a variety of pesticides and it is especially useful for polar and ionic pesticides. However, the most hydrophobic pesticides, which are not ions in solution or not sufficiently polar, may have problems with electrospray.

Overall, the use of ESI techniques for environmental analysis will be increasingly used do to the enormous potential as regards sensitivity and identification of polar and ionic unknown pesticides and metabolites. The combination of LC-ESI-MS-MS, either in a tandem MS system or by using ion trap MS, is extremely powerful and should permit unequivocal identification of pesticides and many unknown organic compounds (today) in environmental samples.

4.4.3 LC-APCI-MS

When LC-APCI-MS is used, the ionization process involves gas-phase ion molecule reactions, which cause the ionization of the analyte molecules under atmospheric pressure conditions. Under APCI-MS, both heat and pneumatic nebulization are applied to evaporate the sample solution and to obtain the spray. APCI-MS is expected to be less dependent on the preionization of the analytes in the sample solution and more suitable for the determination of compounds covering wide polarity ranges including polar and medium or even non-polar analytes. The most relevant parameters to optimize are the capillary voltage and the corona current. The capillary voltage is applied to the entrance of the capillary and it is relative to the nebulizer and spray chamber which are at ground potential. The corona current parameter controls the current (in μA) from the corona discharge needle to the end plate. The field of free electrons that makes up this current ionizes the mobile phase molecules. The ionized mobile phase molecules in turn react with, and ionize, the sample molecules. These two values are slightly different in positive and in negative ionization mode according to the specifications of the mass spectrometer system. The fragmentor voltage affects the transmission and fragmentation of sample ions. In general, the higher the fragmentor voltage, the more fragmentation will occur. In compounds that do not fragment readily, higher fragmentor voltages often result in better ion transmission. The fragmentor voltage gives the ions a "push" that helps them traverse the relatively high pressure region between the exit of the capillary and skimmer. At higher voltage values the maximum structural information is achieved. In a recent study [178] the determination of cyanazine, cyanazine acid and cyanazine amide was carried out by LC-APCI-MS with optimization of the different parameters. It is known that optimum fragmentor voltage is compound dependent and, for this reason, an accurate evaluation of

a wide range of fragmentor values for each one of the compounds studied was performed. In Fig. 4.17 the different pattern in fragmentation for cyanazine and its two metabolites is shown as a function of the fragmentor voltage. In all three cases, the molecular ion
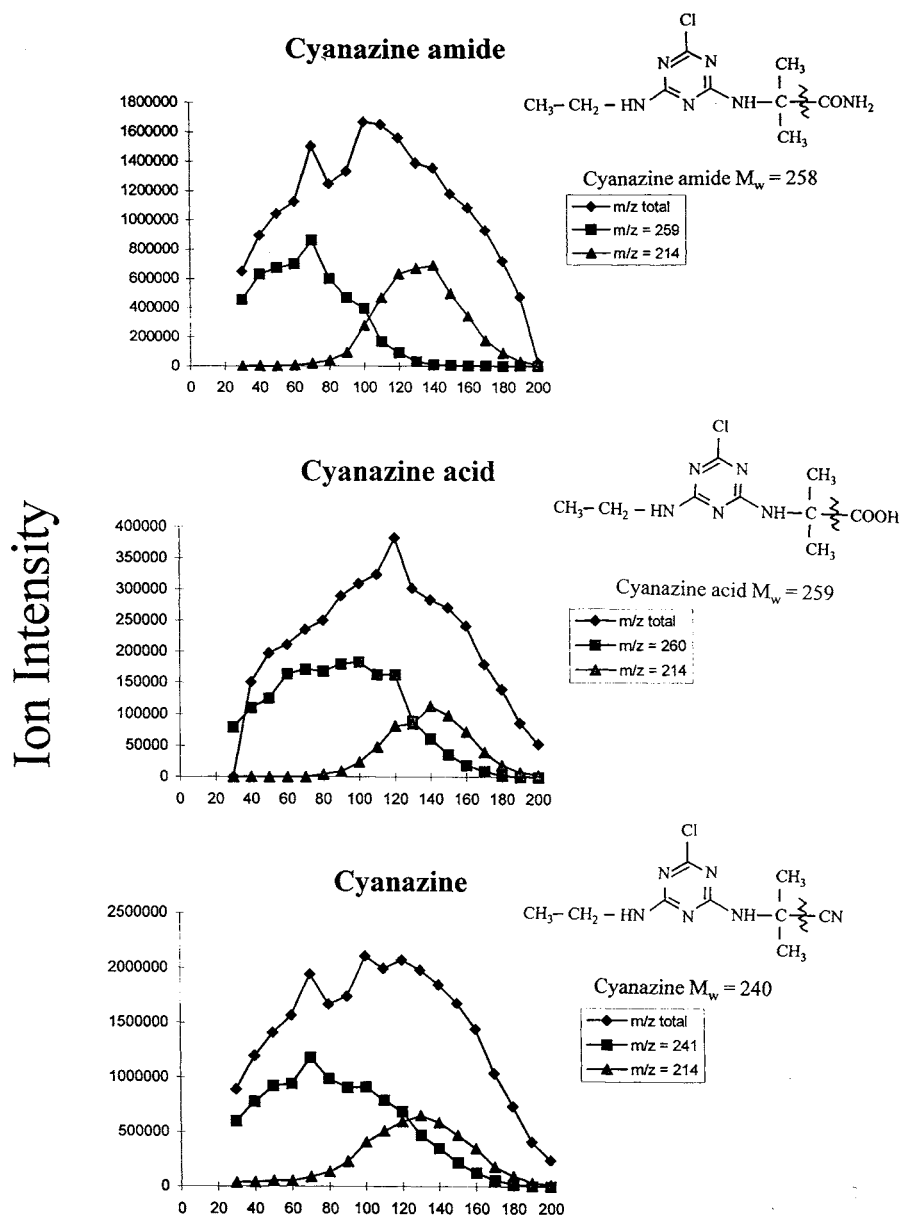
Fig. 4.17. Fragmentation pattern of cyanazine amide, cyanazine acid and cyanizine studied under LC/APCI/MS in Positive mode of operation. LC mobile phase: 40% acetonitrile, 60% water (0.3% acetic acid).

presents its maximum sensitivity at a fragmentor value of 70 V. At higher fragmentor values the sensitivity for the molecular ion decreases with the exception of cyanazine acid which remains constant until 120 V. The main fragment ion obtained was the 214 ion which corresponded to the loss of the amide, carboxylic and cyano group for cyanazine amide, cyanazine acid and cyanazine, respectively. In this sense, the pattern exhibited by the fragment ion was similar in all three cases, showing a maximum sensitivity for fragmentor values around 130–140 V. A fragmentor value of 120 V was chosen for the analysis of groundwater samples in order to identify the major metabolites of cyanazine. At this value the maximum fragmentation without decreasing the sensitivity for the molecular ion was obtained. Table 4.13 reports the typical ions of the three compounds studied in this work in both positive and negative mode of operation. Fragmentor voltages of 70 and 120 V were studied in order to assess the best conditions of detection under LC/APCI/MS. All the compounds studied gave only the molecular ion plus a proton or minus a proton as a base peak under positive and negative mode of operation, respectively. It was observed that, under positive ion conditions, all three presented a major fragmentation at 120 V, which gave both good structural information and enough sensitivity for the pesticides studied. On the other hand, under negative ion conditions, scarce fragmentation was observed for cyanazine acid as compared with positive mode of ionization. The positive mode of operation was chosen for the construction of calibration curves and quantitation of environmental groundwater samples whereas negative mode of operation was only used for further confirmation of positive results, especially for cyanazine acid which presented a higher sensitivity under negative conditions.

   LC-APCI-MS can usually be applied at flow rates between 0.8 and 1.5 ml/min. In the

TABLE 4.12

TYPICAL FRAGMENT IONS AND RELATIVE ABUNDANCE (RA) OF THE HERBICIDE METABOLITES IN HPLC-ESI-MS IN NEGATIVE ION MODE OF OPERATION. CONE SET AT 20 AND 40 V AND CORONA AT 3.1 kV. CARRIER STREAM: ACETONITRILE–WATER–METHANOL CONTAINING 0.3% ACETIC ACID AT A FLOW RATE OF 0.3 ml/min

Compound	20 V			40 V		
	M_n[a]	m/z	RA	M_n[a]	m/z	RA
Acetochlor ESA	314	314	100	314	314	100
Acetochlor oxanilic acid	264	264	100	264	146	100
		146	90		264	70
Alachlor ESA	314	314	100	314	314	100
Alachlor oxanilic acid	264	264	100	264	160	100
		160	30		264	60
		192	10		158	10
Metolachlor ESA	328	328	100	328	328	100
Metolachlor oxanilic acid	278	278	100	278	206	100
		206	10		278	80
2,4-D	220	219	100	220	161	100
		161	40		219	15

[a] M_n, nominal mass.

TABLE 4.13

TYPICAL FRAGMENT IONS AND RELATIVE ABUNDANCES (RA) OF THE HERBICIDES IN LC-MS USING AN APCI INTERFACE, EITHER IN POSITIVE OR NEGATIVE ION MODE OF OPERATION. FRAGMENTOR SET AT 70 AND 120 V. CARRIER STREAM: ACETONITRILE/WATER (30:70) CONTAINING 0.3% ACETIC ACID AT A FLOW RATE OF 0.8 ml/min

Compound	M_n[a]	APCI in PI				APCI in NI			
		70 V		120 V		70 V		120 V	
		m/z	RA	m/z	RA	m/z	RA	m/z	RA
Cyanazine amide	258	259	100	259	15	257	100	257	90
		242	10	242	50	239	20	239	100
				214	100	222	15	222	10
								194	20
Cyanazine acid	259	260	100	260	100	258	100	258	100
				214	50			172	20
								106	15
Cyanazine	240	241	100	241	100	239	100	239	15
		214	7	214	90			212	60
								194	60

[a] M_n, nominal mass.

last few years, several applications as regards the determination of pesticides in water matrices were reported [178–189].

Similarly, as in the case of LC-ESI-MS, on-line SPE coupling with LC-APCI-MS is also feasible. By using such a device it is possible to determine at ppt level most of the pesticides analyzed by preconcentrating only 100–200 ml of water. Table 4.14 shows the calibration graphs constructed by percolating on-line 20 ml of groundwater sample spiked with a solution containing cyanazine and its two metabolites through a PLRP-s cartridge. The curves were linear in the range studied from 0.01 to 1.5 µg/l and the correlation coefficients were higher than 0.98 for all the pesticides studied, thus indicating a good performance of the methodology developed in this work. The LODs were calculated using

TABLE 4.14

CALIBRATION DATA OBTAINED WITH LC-APCI-MS IN TIME-SCHEDULED SIM-PI MODE FOR THE STUDIED PESTICIDES (SPIKED FROM 0.01 TO 1.5 µg/l) AFTER ON-LINE PRECONCENTRATION OF 20 ml OF GROUNDWATER THROUGH A PLRP-S CARTRIDGE

Compound	Calibration equation[a]	R_2	LOD (µg/l)[b]
Cyanazine amide	$Y = 406763 + 2E + 0.7x$	0.9844	0.002
cyanazine acid	$Y = 139926 + 7E + 06x$	0.9933	0.005
cyanazine	$Y = 100388 + 2E + 07x$	0.9974	0.002

[a] Least squares regression equation.
[b] LODs were calculated by using a signal-to-noise ratio of 3 (the ratio between the peak intensity and the noise).

a signal-to-noise ratio of 3 (the ratio between the peak intensity under SIM conditions and the noise). Low detection limits in the ppt level can be obtained due to the high selectivity and sensitivity encountered by the APCI/MS system. On the other hand, APCI/MS detection has been proven to be very selective for pesticides and their metabolites since any or few interferences are encountered under SIM conditions. The combination of solid phase extraction together with mass spectrometry detection has been demonstrated to be a powerful technique for the preconcentration and detection of not only pesticides but also traces of their main metabolites in environmental water samples [178].

Another interesting remark when comparing APCI with ESP, is that, in general, LC-APCI-MS under NI mode is much easier to perform and to "understand" by a researcher with no MS background than LC-ESI-MS NI. For instance, ionization in ESI is much more dependent on the compound to be analyzed than is the case with APCI. So, in the LC separation of acidic herbicides it is necessary first to add acid in the mobile phase and later on, after post-column elution, a neutralization buffer needs to be added to form ions in solution and to facilitate better charging of droplets [161,172].

Finally, the use of LC with a short column combined with APCI-MS-MS was reported to be a good alternative for the fast determination of 17 pesticides in water matrices, achieving successful identification since MS-MS mode can be used and library identification is guaranteed [185,186,189]. In addition, this system can be used to assess rapid degradation of pesticides after irradiation using a xenon lamp and it can also be used for unequivocal identification of the metabolites formed.

In summary, we should indicate that LC-APCI-MS will be the method of choice for most of the environmental applications, mainly due to easy use since it can be combined with most LC equipment available using conventional flow rates. By either PI or NI the characterization of a large number of analytes is feasible. The combination with either off-line or on-line SPE devices, permits the automation of the determination of pesticides in water matrices and the achievement of LODs which comply with the most stringent regulations of pesticide residues in water matrices.

4.5 CONCLUSIONS

In this chapter we have summarized the different sample handling methods followed by LC determination of pesticides and their transformation products in water samples. In recent years many methods of determination involving several groups of pesticides were developed and we can say that nowadays methodologies based on LC are well established. Still, problems remain in the simultaneous determination of parent pesticides and their polar transformation products. Many times a second sample handling procedure needs to be performed in order to analyze the more polar metabolites. We should mention that new metabolites are being found and more metabolites will be found in the coming years. This is certainly an area of growing interest. With the advent of LC-MS methods based on atmospheric pressure, it has been possible to identify new metabolites. This will be enhanced when more MS-MS methods combined with tailor-made specific sample handling procedures are performed together with the synthesis of new metabolites

As regards sample handling methods, new areas are being developed, like the routine implementation of immunosorbents – in this case the use of monoclonal antibodies will be

preferred since reproducibility of the immunosorbents is guaranteed – and molecular imprints. Such solid phase extraction materials will permit the enrichment of any target analyte that is present in a water matrix. But still problem definition will be needed and it is obvious that the experience of the laboratories working in the environmental area will be needed in defining which target pesticides and metabolites will need to be analyzed in specific water samples

Finally, as regards the analytical determination, LC and LC-MS are well developed and routinely used. New developments will need to take place in the areas of capillary electrophoresis and capillary electrochromatography. Such techniques permit high separation power and selectivity which is an advantage when analyzing environmental samples. A disadvantage is still the sensitivity and the difficulties in coupling these techniques to MS detectors.

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Chapter 5

Environmental applications of gas chromatography-atomic emission detection

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CONTENTS

5.1 INTRODUCTION

Dealing with real-life samples is still a challenging goal for the analytical chemist, especially the chromatographer. One problem is resolving adjacent and/or co-eluting components, another is the identification and characterization of separated solutes. Since the introduction of gas chromatography (GC) half a century ago, a great number of detectors have been employed. A detector should have sufficient sensitivity and selectivity, and the possibility to predict structure. So-called universal detectors respond to changes induced by the solutes in the physical property of a characteristic mobile phase. These are rather insensitive and provide little structural information about the separated species. Detectors that respond to some physico-chemical property of the separated components are known as selective or specific detectors and they provide chemical structural information.

Analytical spectroscopy has, therefore, gained an increasingly important role in chromatographic detection. It is capable of generating spectral data for each individual compo-

nent. The development of fast scanning spectrometers, i.e. Fourier transform techniques, has been quite effective for their employment in chromatography as a detection device. This type of detection system, such as mass spectrometry (MS), infrared spectroscopy (IR) and atomic emission spectroscopy (AES), is considered to be "property selective", "structure or functionality selective" and "element selective", providing solute characterization based upon these various physico-chemical features [1,2].

Among all available analytical techniques, atomic spectroscopy (AS) is regarded as one of the most suitable for elemental analysis. The potential combination of AS with chromatographic systems, therefore, was initially not well recognized, as AS is mainly used for inorganic species whereas chromatography is mainly used for organic compounds. However, the development of analytical technology has been an effective driving force for hyphenation of lots of instrumentation, one of the components usually being a form of chromatography.

Atomic absorption spectroscopy (AAS), atomic fluorescence spectroscopy (AFS), flame emission spectroscopy (FES), and atomic emission spectroscopy (AES) have been interfaced with chromatographic systems [3,4] to date. The capability for simultaneous multi-channel determination, wide dynamic range and relatively high sensitivity and selectivity are advantages of AES. This technique, especially when it utilizes plasma, has therefore been the focus of attention for chromatographic interfacing. The high temperature of plasma produces sufficient energy to break down all molecular species to their constituent atoms and related ions, which are excited by the plasma energy and subsequently emit radiation at a wavelength characteristic of the specific element.

In this chapter, the GC-AED system based on the helium plasma will be explained and its environmental applications to metal species and organic compounds will be reviewed.

5.2 INSTRUMENTATION

5.2.1 General

Among the available AS techniques, the microwave-induced plasma (MIP) is mostly used in conjunction with GC. Helium plasma can be generated within a cavity which concentrates energy from a microwave source into a quartz capillary tube called a discharge cell. It is possible to operate the plasma at atmospheric or reduced pressures. The available power levels are somewhat lower than for direct current plasma (DCP) or inductively coupled plasma (ICP), making its operation easier and helium consumption much lower. However, since the size of the MIP is reduced, power densities are rather similar. The helium MIP generates a lower plasma temperature but due to the nature of its chemistry, high spectral emission intensities are produced for many elements among the non-metals.

In 1989, a detection system for capillary GC that employed multi-channel atomic emission detection (AED) with a helium MIP was introduced. Eluted substances are converted into atoms and the radiation emitted by excited atoms is simultaneously detected by a photodiode array (PDA) over a portion of the spectrum [5]. Theoretically, the AED response for a compound is an elemental phenomenon and independent of the original molecular structure. Therefore, the analytes of interest can be quantified on the basis of data from a single elemental calibration curve using one internal or external
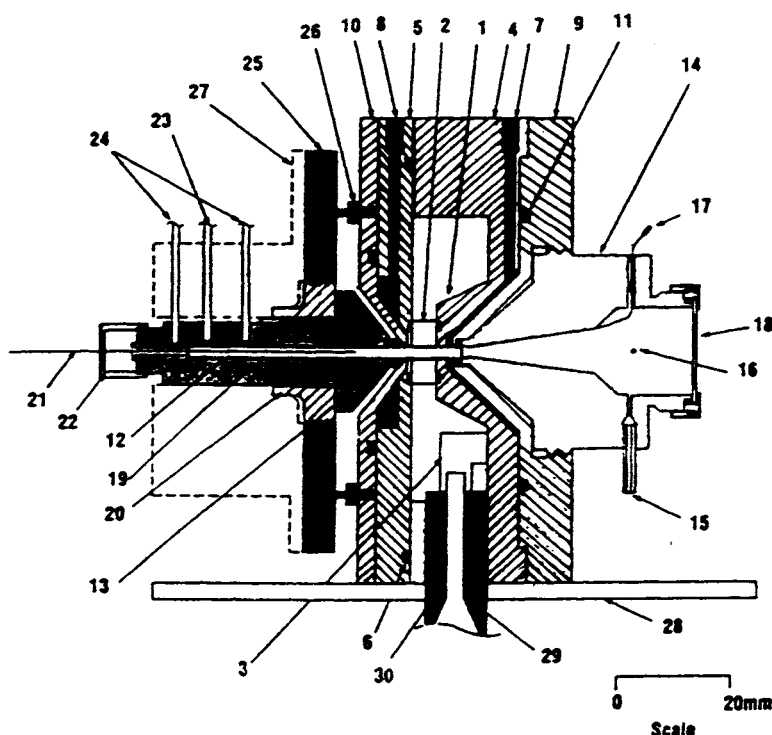
Fig. 5.1. Reentrant cavity: (1) pedestal, (2) quartz jacket, (3,4) main cavity body, (5) cavity cover plate, (6) gasket, (7,8) cooling water inlet and outlet, (9,10) water plates, (11) O-ring, (12) silica discharge tube, (13) polyimide ferrule, (14) exit chamber, (15,16) window purge inlet and outlet, (17) sparker wire, (18) window, (19) gas union, (20) threaded collar, (21) column, (22) capillary column fitting, (23) make-up and reagent gas inlet, (24) purge flow inlets, (25) stainless steel plate, (26) standoff, (27) heater block, (28) mounting flange, (29) brass center conductor, (30) PTFE coaxial insulator.

reference standard. The helium MIP has been the most commonly used detection device for monitoring non-metal species, as for many of these elements, helium metastable energy carriers demonstrate inadequate collision energy transfer for sufficient excitation.

5.2.2 The interface

Beenakker [6] developed an atmospheric pressure TM_{010} cylindrical resonance cavity, shown in Fig. 5.1, which is the commonest interface for GC detection. As outlet from GC is usually at atmospheric pressure, interfacing between these two instruments becomes simple. This is especially true when capillary columns are used and the dead volume is in the microliter range. Heating is essential to prevent any condensation along the interface line. Helium make-up gas or other reagent gases can be introduced within the transfer line to minimize peak broadening and optimize plasma performance. Performing the GC-MIP with a tangential flow torch [7] leads to self-centering plasma with enhanced emission and higher sensitivity, at the expense of high volumes, i.e. many liters per minute, of helium

gas. For conjunction of GC and reduced pressure plasma, it was necessary to reduce the interface chamber to a pressure of about 1 Torr. Little degradation in peak efficiency for packed columns can be observed but the broadening of capillary peaks to some extent is inevitable.

5.3 APPLICATIONS

Since the GC-AED introduction in the last decade, like some other analytical tools, e.g. GC-mass spectrometry (GC-MS), liquid chromatography-MS (LC-MS), it has been used in environmental studies. Many aspects of this quite important field of research have been the focus of attraction which can be divided into two major categories, inorganic species and organic compounds. Pedersen-Bjergaard and Greibrokk [8] presented an extensive review on environmental applications of capillary GC coupled with AED emphasizing both the commercial and laboratory-built systems. They devoted much of their attention to three categories of element-selective detection of non-metal as well as metal pollutants, identification of contaminants and sample preparation consideration.

5.3.1 Metal heteroatom species

The widespread use of organometallic compounds and their subsequent release into the environment has been a focus of environmental concern in the last decades [9,10]. Among all types of species, metals like lead, tin and mercury are of most importance. For lead, the major part of the environmental burden is due to the use of tetraalkyllead compounds, mainly tetraethyl, tetramethyl and some related mixed compounds, which are all toxic substances, and applied as antiknock additives to petrol. Organotin compounds have even more versatile applications. In particular, the use of triorganotins (R_3SnX) in antifouling paints (R = butyl, phenyl) and pesticides (R = phenyl, cyclohexyl) has caused environmental problems. Industrial usage of mercury compounds, i.e. in seed dressing, fungicides and pesticides, and in paint products, have raised concern about toxicity.

Owing to the inherent advantage of elemental response of AED, inorganic species, especially the metal-containing compounds, were among the first groups of chemicals that were determined in various environmental samples. Since the GC part of the system performs the separation of analytes, derivatization of metal species into a more volatile compound is, therefore, essential and in almost all cases chemical modification of analytes should be carried out prior to the GC-AED analysis.

Talmi and Norvall [11] employed a reduced-pressure GC-MIP for the determination of arsenic and antimony with detection limits of 20 and 50 pg in environmental samples. They derivatized triphenylarsine and triphenylstibine and measured these modified analytes at 228.8 and 259.8 nm, respectively.

An environmental analysis of metallic heteroatom compounds was employed for the specific determination of lead and carbon in trialkyllead chlorides extracted from an industrial plant effluent [12]. Butyl Grignard reagent was used as a derivatizing agent to produce trialkylbutyllead compounds, which are amenable to analysis by GC. The selectivity of AED was clearly shown by eliminating the interference from the high level of carbon-containing compounds, which prevented any useful qualitative and quantitative

measurements of trialkyllead compounds by GC-MS and GC-ECD. Applying the latter devices requires extensive clean-up processes and loss of analyte is more likely to occur.

In a comparison study of reduced- and atmospheric-pressure MIP systems, a detection limit of 1 pg for mercury, using the latter system, was obtained by Olsen et al. [13] with a selectivity over carbon of 10 000.

Ultratrace speciation of organolead compounds in aqueous environmental samples, i.e. tap water and polar snow, using a diode-array detection system was carried out by Lobinski and Adams [14]. They achieved sub-ng/ml detection limits for organolead compounds applying preconcentration in a Tenax-packed liner [15]. A volume of 100 ml of the sample was sufficient for the analysis and no evaporation was necessary prior to the sample introduction into the GC-AED. Sample preparation involved extraction of dicarbamate complexes of ionic organolead species into hexane followed by their propylation using a Grignard reaction. A 25-ml volume of the derivatized extract was injected on to the Tenax-packed liner. The design of the liner and the type of packing had taken place considering the effective separation of picogram amounts of the analytes from a bulk of solvent that could be ten orders of magnitude or even more than that of the analyte. In addition, the liner should permit the instantaneous release of the trapped analytes on increasing the temperature, avoiding spreading and/or tailing of the chromatographic peaks. The Tenax packing was found to be more efficient, with no losses for some propylated compounds. The peaks were sharp and no discrimination for higher boiling compounds was observed. Figs. 5.2 and 5.3 show GC-AED chromatograms of a mixture of propylated organolead standards obtained with and without the employment of a Tenax-packed liner. Before rapid, linear heating of the liner, the solvent was purged away at room temperature by a stream of helium while the analytes were trapped on to the sorbent. As the optimum temperature for the solvent removal depends upon its volatility and that of the analytes some losses of Me_3PbPr occurred at 30°C and the losses became increasingly significant at higher temperatures. For butylated compounds a temperature of 30°C was still acceptable. A minimum temperature of 20°C was found to be essential to remove 20
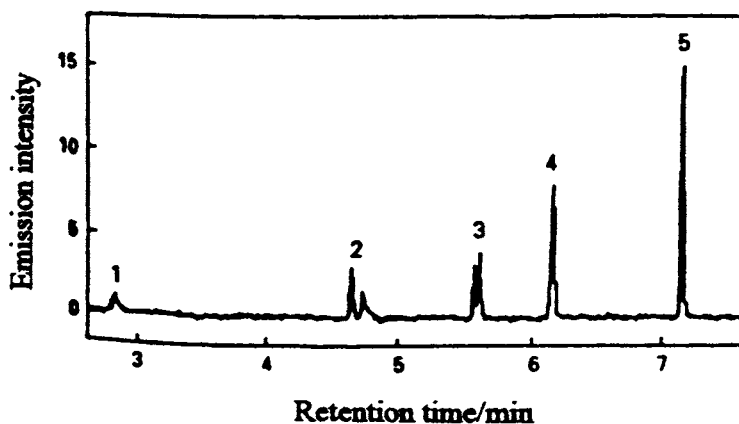
Fig. 5.2. Chromatogram obtained using GC-AED of a mixture of propylated organolead standards (3–4 pg each) obtained with unpacked liner after 60 s of solvent venting. (1) Me_3Pb^+; (2) Me_2Pb^{2+}; (3) Et_3Pb^+; (4) Et_2Pb^{2+}; (5) Pb^{2+}.
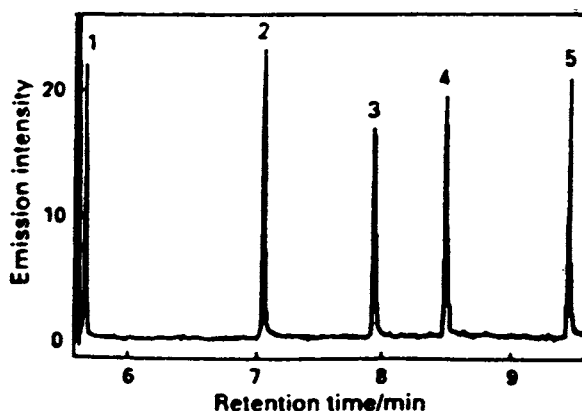
Fig. 5.3. Chromatogram obtained using GC-AED of a mixture of propylated organolead standards (3–4 pg each) obtained with Tenax-packed liner after 60 s of solvent venting. (1) Me_3Pb^+; (2) Me_2Pb^{2+}; (3) Et_3Pb^+; (4) Et_2Pb^{2+}; (5) Pb^{2+}.

ml of hexane in 1 min. Increasing the volume injected to 25 ml required more time for the effective solvent removal. A time of 2 min for solvent purging at 20°C was then quite necessary. The effect of the solvent venting temperature on the recovery of the analytes is shown in Fig. 5.4. The precision of method was 5% at the 1 ng/ml level with 90% recovery.

A comprehensive GC-AED method [16] for the determination of mono-, di-, tri-, and some tetraalkylated organotin compounds in water and sediments employing diethyl-
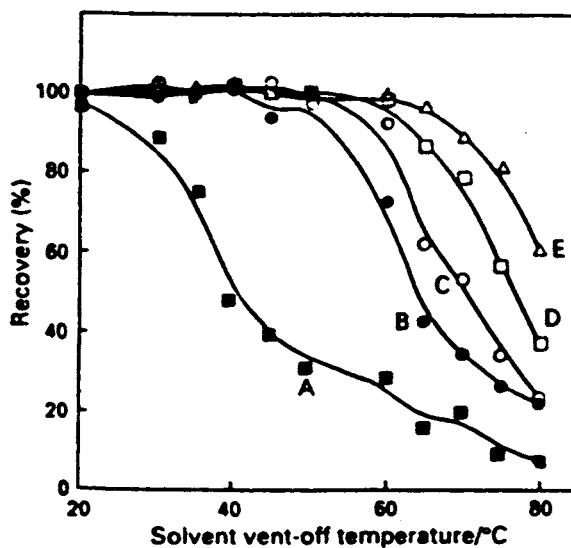
Fig. 5.4. Effect of the temperature of solvent removal on the recovery of propylated organolead species. Venting time, 60 s; helium purge flow rate, 100 ml/min. (A) Me_3Pb^+; (B) Me_2Pb^{2+}; (C) Et_3Pb^+; (D) Et_2Pb^{2+}; (E) Pb^{2+}.

dithiocarbamate extraction and subsequent Grignard derivatization was developed. Ionic organotin species were extracted as diethyldithiocarbamates into pentane and, after evaporation, dissolved in a small volume of octane and derivatized with pentylmagnesium bromide to give a solution amenable for GC analysis. The absolute detection limit was 0.05 pg, and the calibration curve was linear over three orders of magnitude. An independent analysis by GC-AAS confirmed the accuracy of the developed method. The analytical response was independent of the temperature necessary for eluting the compounds, therefore, allowing for internal standard calibration. A method to determine the empirical formula of the detected compounds was also proposed. Although the method could not match the sub-percent precision of classic microanalysis it demonstrated some advantages like having the possibility of carrying out measurements on-line and utilizing considerably lower amounts of sample, i.e. picograms instead of milligrams, necessary for the analysis. It provided a means for identification of unknown chromatographic signals. The same derivatization procedure for organotin species was applied after their sorption from water samples using C_{18}-packed microcolumns and extraction disks [17]. This led to the development of a semi-automated system for on-line preconcentration/derivatization of organotin compounds in river water samples by GC-AED. The applicability of the method to the determination of organotin species in real life samples was demonstrated by the analysis of many river water samples from the river Scheldt estuary. Monobutyltin, dibutyltin and tributyltin were detected in all the samples analyzed at concentrations in the ranges 10–140, 8–67 and 4–11 ng/l, respectively. Although the inorganic tin peak appeared as a rather small signal quite nice chromatograms for real-life samples were achieved. A 10–50 ml water sample was sufficient for the analysis and a detection limit of 0.1 ng/l could be obtained. The developed procedure was compared with the manual LLE procedure and the results agreed within 10–15% for low ng/l concentrations. Comparison of GC-MSD and GC-AED for the determination of organotin compounds in water, sediment and suspended matter was also studied [18]. All organotin compounds which are used in biocides, i.e. tribytyltin, triphenyltin, tricyclohexyltin and fenbutatin oxide, were selected to be studied. Apart from the mentioned list, five degradation products were also determined in water samples. Both methylation and pentylation were employed for derivatization; the latter was found to be more efficient. The organotin compounds and their related degradation products were determined at levels of 1–10 ng/l using 200-ml water samples. Earlier, another group [19] carried out a comparison study for identification and determination of organotin compounds in water and sediment by GC-AED. The quality criteria for the quantified data were found to be dependent on the matrices and the tin species, however, the recovery rates were higher for aqueous samples than for sediment. The kinetics of recovery experiments showed that parts of the organotin compounds were irreversibly adsorbed on solid phases.

Methylated species of tin, lead and mercury are rather important in environmental studies, as they can be both anthropogenically introduced and form naturally in the environment via so-called biomethylation processes. The high toxicity of these compounds has encouraged the development of accurate and sensitive analytical methods. Sample preparation for alkyltin, -lead and -mercury usually requires extraction, enrichment and derivatization steps, often under conditions strongly differing for each element.

A method for the simultaneous determination of mercury species at sub-ng/l levels in natural water was described by Emteborg et al. [20]. They reduced the sample volumes to

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less than 1 1 and facilitated the simultaneous determination of methylmercury and inorganic mercury to be applied to sea and humic-rich water samples. Mercury-containing species were trapped on a dithiocarbamate resin-loaded microcolumn and eluted with acidic thiourea. After neutralization, the eluted species were extracted in toluene as diethyldithiocarbamate complexes and butylated with Grignard reagent. The chemically modified species were then separated and determined by GC-AED. The developed method was applicable with detection limits of 0.05 ng/l for methyl- and ethylmercury and 0.15 ng/l for inorganic mercury in 0.5-l samples, in sea and fresh water, but failed in the presence of high concentrations of humic substances.

Ceulemans and Adams [21] described the optimization of a purge-and-trap injection (PTI)-GC-MIP-AED system for the simultaneous sample preparation and multi-element detection of inorganic mercury and methylated tin, lead and mercury species in water at trace levels. They performed the chemical modification of analytes using sodium tertraethylborate (NaBEt$_4$). This reagent can be used for simultaneous derivatization of tin, lead and mercury compounds in metal speciation analysis [22]. The ionic species were volatilized from the aqueous sample after ethylation with NaBEt$_4$ in an acetate buffer medium of pH 5 and preconcentrated on a capillary cryogenic trap. Desorption was applied by linear heating of the trap, followed by GC-MIP-AED analysis. Consuming a 10-ml sample volume, detection limits of 0.15, 0.20 and 0.60 ng/l for methylated tin, lead and mercury species, respectively, and 2 ng/l for inorganic mercury could be obtained. The developed method was successfully applied to the analysis of two water samples; the results are presented in Table 5.1 and show simultaneous detection of methyltin, mercury and methylmercury species at low ng/l levels. No methyllead species were observed in the river water while they were present in the soil run-off water. Methyltin species and methylmercury could not be detected in the latter sample. The mercury, tin and lead traces of the river water sample are shown in Fig. 5.5.

Lately, speciation of organotin and organolead compounds in drinking water by GC-AED was performed [23]. Water samples were extracted with 30 ml pentane at pH 5 and 9 for organotin and organolead speciation, respectively. The extracts were then alkylated with pentylmagnesium bromide and butylmagnesium chloride, respectively. After clean-

TABLE 5.1

RESULTS OF THE ANALYSIS OF RIVER AND SOIL RUN-OFF WATER

Compound	Concentration (ng/l)[a]	
	River water	Soil run-off water
Me$_3$Sn$^+$	4.08 ± 0.29	<DL[b]
Me$_2$Sn^{2+}	0.50 ± 0.06	<DL
MeSn^{3+}	0.31 ± 0.04	<DL
MeHg$^+$	4.82 ± 0.55	<DL
Hg^{II}	4.40 ± 0.51	11.3 ± 1.0
Me$_3$Pb$^+$	<DL	14.2 ± 0.6
Me$_2$Sn^{2+}	<DL	1.66 ± 0.12

[a] Mean ± standard deviation ($n = 4$), expressed as metal.
[b] <DL: below detection limit.
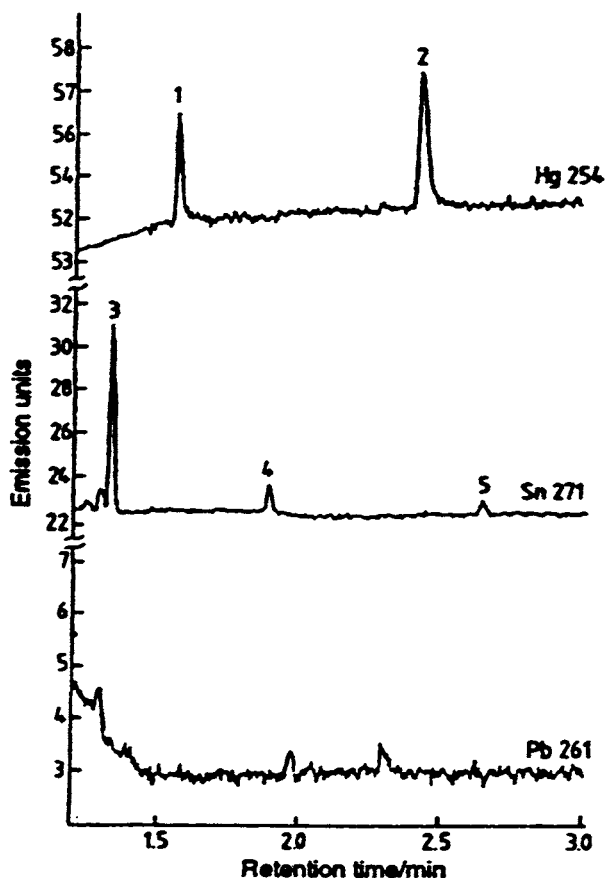
Fig. 5.5. Chromatogram for a sample of river water: (1) MeHg$^+$; (2) Hg^{II}; (3) MeSn$^+$; (4) MeSn^{2+}; (5) MeSn^{3+}.

ing-up and evaporation, the extracted sample was analyzed by GC-AED. Organotin compounds were detected up to 22 ng Sn/l and 43.6 ng Sn/l for methyltin and butyltin, respectively.

The use of microwave irradiation for the extraction of metal species, causing rapid and simple sample preparation, has been extended to the GC-AED analysis. In a recent work [24], monobutyltin, dibutyltin and tributyltin in two standard sediments and in fish tissue were extracted in a 60-W microwave field for 3 min. After ethylation, the organic phase was analyzed by GC-flame photometry or GC-AED.

5.3.2 Non-metal heteroatom species

GC-AED with a helium MIP has been the most used detection device for monitoring non-metal species, as for many of these elements, helium metastable energy carriers demonstrate inadequate collision energy transfer for sufficient excitation. Thus, GC-MIP-AED has been extensively applied to the analysis of organic pollutants for all

types of environmental samples. Many important classes of these pollutants are discussed here.

5.3.2.1 Volatile organic compounds

Volatile organic compounds (VOC) in atmospheric samples include those compounds that have a sufficient vapor pressure at ambient temperatures to keep them in the vapor phase. Compounds having boiling points well below ambient temperature such as ethane, vinyl chloride, and acetylene are included in this class, as well as those which are volatile liquids at room temperature, i.e. benzene, hexane, octane or acetone. The class ranges up to liquids which boil in the vicinity of 250°C [25].

The first application of atmospheric pressure GC-MIP to environmental samples was halogen-specific detection of haloorganics in drinking water [26]. Although the electron capture detector (ECD) is more sensitive than the MIP for polyhalogenated compounds, its response is very much molecular-structure dependent while the MIP response is only due to the presence of each halogen. After applying extraction and purge-and-trap techniques, sub-ppb detection and quantification of halomethanes was easily obtained.

The analysis of the VOC in urban air usually reveals many components which can be attributed to gasoline, as well as those compounds being emitted by other local sources. The analysis of this class of compounds in atmospheric samples usually involves collection of the sample, concentrating the VOC from a large enough quantity of air to obtain sufficient material to detect, and, finally, separating and identifying the individual compounds. The separation and identification step is usually carried out by GC or GC-MS, but the sampling and concentration steps may be laborious and time consuming and can be performed in several different manners. The sample can be seriously biased if these steps are not carefully designed and carried out.

Some volatile heteroatom organic compounds which are present in air were analyzed by GC with thermal desorption cold-trap (TCT) injection and AED and mass-selective detection (MSD) [27]. A mini pump was used to pass a known volume of air, e.g. 1 l through a glass sample tube packed with a Tenax GC held in place with small plugs of silica wool. The sample tube was inserted in the TCT where the trapped species could be desorbed by applying heat and maintaining the temperature at 250°C for 10 min at a flow of 1 l/min. The compounds were swept by the carrier gas stream to the cold trap already cooled to −120°C for 5 min. GC injection was subsequently performed by flash heating the cold trap to 250°C for 5 min. Sampling efficiency studies revealed that 1000-ml sampling volumes could cause retention of compounds less volatile than toluene by the Tenax sorbent, and the desorption conditions chosen resulted in complete transfer of analyte from the sampling tube to the cold trap. The total ion current (TIC) chromatogram obtained from an urban air sample taken in Tokyo showed that most of the peaks were due to the presence of aliphatic or aromatic hydrocarbons such as *n*-hexane, benzene, toluene, xylenes and naphthalene. In addition, four chlorinated compounds, 1,1,1-trichloroethane, trichloroethylene, tetrachloroethylene and *p*-dichlorobenzene, were also identified. Fig. 5.6 shows carbon- and chlorine-channel traces obtained from urban air by TCT-GC-AED analysis recorded at 496 and 479 nm, respectively. Unfortunately this short communication includes no quantitative data regarding these environmentally hazardous compounds.

Gerbersmann et al. [28] established a method for the determination of 13 volatile sulfur
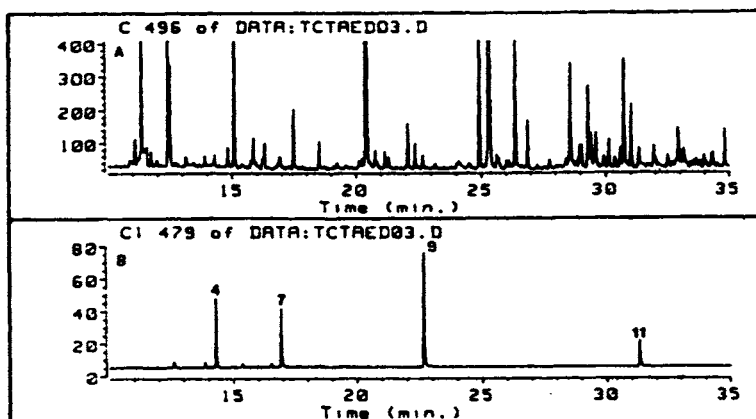
Fig. 5.6. Sections of chromatograms obtained by TCT-GC-AED analysis of an urban air sample: (A) carbon-specific chromatogram (496 nm); (B) chlorine-specific chromatogram obtained simultaneously (479 nm).

compounds, i.e. dimethyl sulfide (DMS), dimethyl disulfide (DMDS), carbon sulfide and other sulfides and thiols, in water samples. The analytes were stripped from the sample by a flow of helium and preconcentrated in a capillary cryogenic trap. They made a flow system to automate the sample delivery (Fig. 5.7). A peristaltic pump was used to deliver the contents of a sample loop (0.5 or 1 ml) and a spiked loop (100 μl) to a purge-and-trap injector. The PTFE tubing was passed through a septum into the purge vessel. The sample and the standard solution were kept in an ice bath. To start the automated sample delivery the cryogenic trap was cooled down and both six-port switching valves were switched into the "load" position. The sample loop was loaded with the sample while water still passed through the spike loop. The spike loop was started by switching valve 3 to "standard solution" to minimize the consumption of standard solution. After filling both loops, valve 1 and then valve 2 were switched into the "inject" position so that water can pass through the sample loop to the spike loop forcing their contents out via the pump into the purge
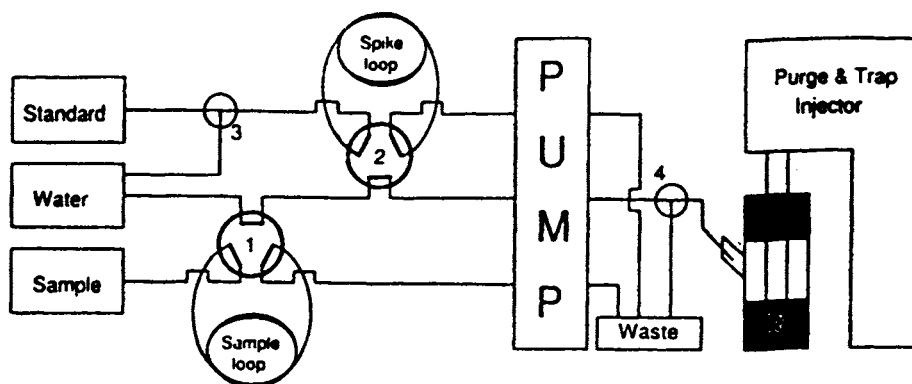
Fig. 5.7. Automated sample delivery system (1–4 are switching valves).
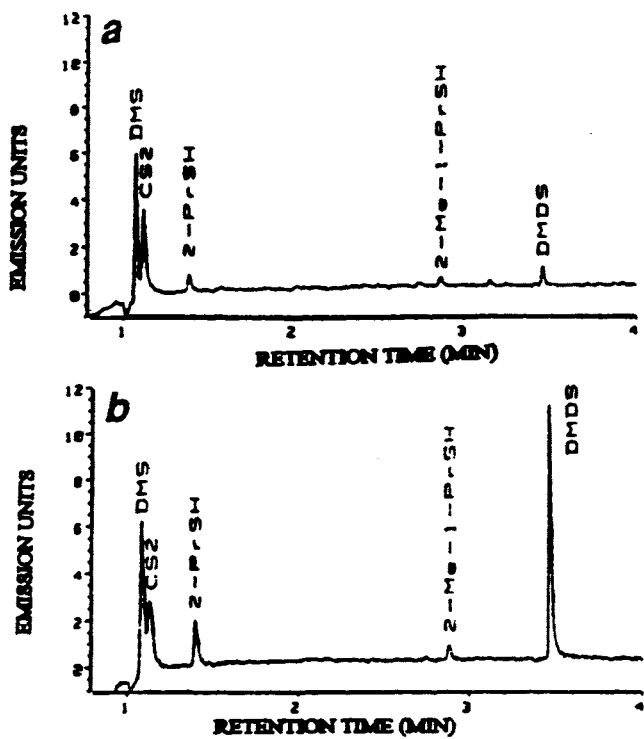
Fig. 5.8. Chromatogram of 20 ml of lake water sample; (a) analyzed directly after sampling; (b) after 24 h.

vessel. This was followed by GC-MIP-AED analysis of samples. Detection limits ranged from 8 pg (0.4 ng/l) for ethanethiol to 17 pg (0.9 ng/l) for DMDS. The developed method was applied to the quantitative analysis of fresh water and sea water and after slight modifications also to different beer types and coffee powder. Fig. 5.8 shows sulfur traces of 20 ml of a lake water sample, (a) immediately after sampling and (b) 24 h after
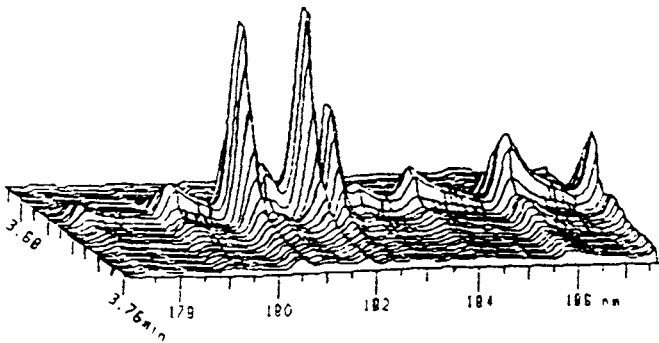
Fig. 5.9. 3D snapshot of a sulfur peak in coffee.

sampling. While the signals of DMS, CS_2 and 2-methyl-propanethiol were not altered, a marked enhancement for the signals of 2-propanethiol and DMDS was observed. Repeating the analysis on successive days revealed that at the high temperature of the laboratory microbial activity in the water sample, which contained many microorganisms and green algae, was notably increased compared to the original activity at normal water temperatures in the lake. Analysis of water samples taken from the North Sea demonstrated that only DMS, CS_2 and DMDS could be detected. The sulfur trace of 0.5 g coffee extract indicated that some volatile sulfur compounds including ethanethiol, DMS, CS_2, thiophene and DMDS are present. The presence of sulfur was confirmed by taking snapshots of the signals as shown in Fig. 5.9 in which three characteristic emission lines, i.e. 180.7, 182.0 and 182.7 nm, can be easily recognized. The mean reproducibility was 3.4% and low sample volumes were needed.

5.3.2.2 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) have quite unique physical and chemical properties, such as remarkable thermal stability, low flammability, high electrical resistivity and suitable viscosity–temperature relationships. They are lipophilic industrial pollutants which show bioaccumulating and biomagnifying properties. Since 1922, this type of mixtures has been extensively used for many industrial applications. Although many countries around the world have banned the use of PCBs since the late 1970s, they are still major environmental pollutants owing to careless waste disposal and their high stability and bioaccumulation potential. Several reports have shown that non-ortho substituted PCBs (IUPAC No. 77, 126, and 169) are the most toxic congeners, the mono-ortho substituted PCBs 60, 105, 110, 114, 118, 156, 157, and 167 are moderately toxic, while the remaining 197 congeners are expected to be relatively non-toxic. Environmental risk assessments for PCBs should, therefore, include separation and quantitation of the individual congeners rather than considering "total PCB" residue levels.

GC-ECD and GC-MS are the two methods which are currently used for the determination of PCBs and all other highly halogenated environmental contaminants. However, GC-ECD does not provide structural information while the sensitivity of electron impact (EI)-MS is lower than that of GC-ECD [29]. Negative chemical ionization (NCI)-MS is a sensitive and selective method for monitoring organochlorine chemicals and has been applied to the determination of PCBs as well. GC-AED, on the other hand, provides excellent elemental selectivity but suffers from a lack of sensitivity. GC-AED, therefore, has been used for PCB analysis in few instances [30,31].

Basic parameters associated with practical application of GC-MIP-AED in the determination of seven "indicator" PCBs in biotic matrices were evaluated and the resulting data were compared with those obtained by "classical" approaches [32]. The limit of detection for chlorine, measured at 479 nm, was found to be 0.54 pg/s. Taking into account the conditions used, this value corresponds approximately to 0.15 mg/kg of the respective congener in fat. The linearity of the detector response was found to be satisfactory and the RSD of repeated injections for the lowest concentration level of 0.5 ng of PCB per injection was 10–35% depending on the number of chlorine atoms in each individual congener. No significant difference between the quantitative date obtained from the AED and those recorded with a conventional ECD was observed (Table 5.2). Although

TABLE 5.2

DETERMINATION OF PCBS (mg/kg OF FAT) IN VARIOUS MATRICES BY GC-MIP-AED

Analyte	Method	Origin of fat sample		
		Fish from accidentally polluted area	Fish from unpolluted area, monitoring site	Beef from contaminated cow-shed
Sum of 7 indicator	GC-ECD	22.41	1.86	3.16
PCBs	GC-MIP-AED[a]	20.17	2.04	2.91
Total PCBs	GC-MIP-AED[a]	137.25	5.67	3.48
Chlorine contained in PCBs	GC-MIP-AED[a]	60.39	2.67	2.10
Contribution of PCB chlorine (%W) to total PCBs	GC-MIP-AED[a]	44.0	47.1	60.3

[a] Calculations are based on data obtained on carbon and chlorine channels.

ECD is a rather sensitive detection device it can respond to many other even non-chlorinated species while the element selectivity of MIP-AED makes it possible to avoid the bias resulting from the non-specific detection of interferents. Using a real-time multi-point background correction method included in the MIP-AED software facilitates the reduction of undesirable signals. Fig. 5.10 shows traces of contaminated fish oil recorded from the carbon and chlorine channels. No interfering signals were recorded in the chlorine trace at retention times corresponding to the elution of co-extracts detected in the carbon channel. A snapshot portion of the spectrum produced by PDA at the maximum of the peak marked with an asterisk is shown in the insert in Fig. 5.10b. The atomic emission lines at 479.5, 481.0 and 481.9 nm clearly confirm the presence of the chorine atom in this compound. The high selectivity of chlorine detection and also the simultaneous possibility of recording a multi-element profile for a sample are the main advantages of GC-MIP-AED for the determination of PCBs in biotic matrices.

The quantitative aspects of capillary GC-AED were extensively evaluated for the analysis of PCBs in technical mixtures and in environmental samples [33]. Special attention was paid to the quantitation by universal calibration of individual congeners, the estimation of total PCBs and calculation of the percentage by weight of chlorine in PCB residues. For comparison of sensitivity, The experiments were carried out with a commercial GC-AED system and a laboratory-built GC AED system based on a 350-kHz on-column RF-plasma, utilizing an emission line at 837.6 nm for chlorine selective detection. As can been seen in Table 5.3, with the commercial atomic emission detector, standard solutions of PCBs were detected down to the 250–400 pg level, whereas detection limits with GC-ECD were in the range of 0.02–0.04 pg. Although signals were close to the detection limit, the baseline was very stable with GC-AED, while the baseline fluctuated considerably with GC-ECD. On the other hand, the present version of the commercial GC-AED system responds only to relatively concentrated PCB extracts, demanding large sample amounts in cases of low PCB levels. Considering recent instrumental development by sustaining the plasma inside the end of the capillary GC column and reduction of make-up gas consumption, due to the small volume of the plasma cell, the GC-AED detectability
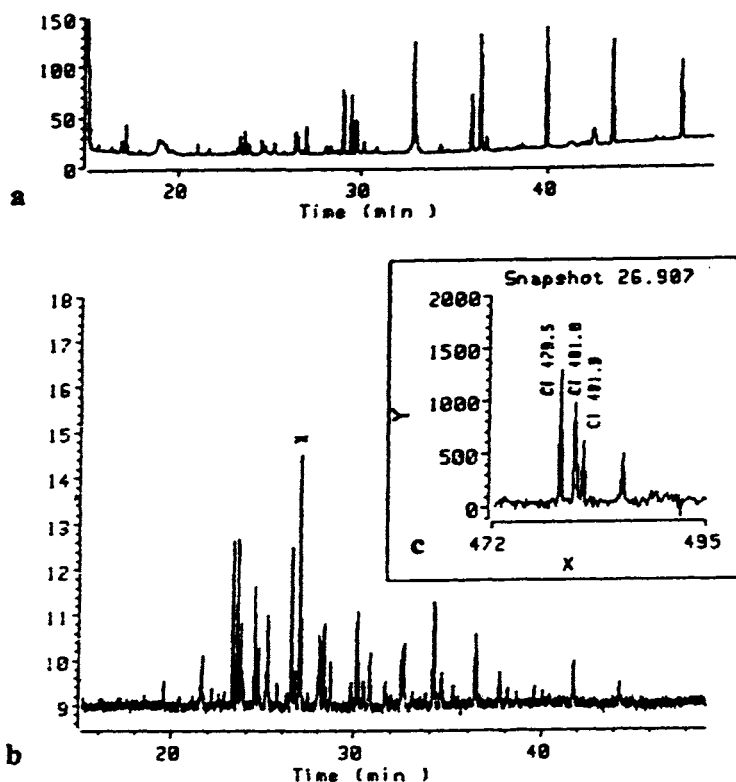
Fig. 5.10. GC-MIP-AED of fish oil extract. Injection corresponds to 2.5 mg of original sample, (a) recorded on carbon (C-496) channel; (b) recorded on chlorine (Cl-479) channel.

was significantly improved. When PCBs were monitored from a laboratory-built on-column plasma system, the detection limits were enhanced by a factor of approximately 30, and the applicability of GC-AED for trace analysis was extended (Fig. 5.11). Although PCB detection limits were higher with GC-AED than with GC-ECD, the GC-AED technique was quite attractive for PCB investigation and provided significant simplification for PCB quantitation. Later on, in a similar study the PCB detection limits obtained from the GC-AED, GC-MS (recorded in selected ion monitoring, SIM, mode) and GC-ECD were

TABLE 5.3

COMPARISON OF DETECTION LIMITS FOR PCBs 31, 110, AND 185

PCB no.	Number of Cl atoms	Detection limit (pg)		
		Commercial GC–AED	On-column GC–AED	GC–ECD
31	3	400	12	0.04
110	5	263	9	0.03
185	7	248	8	0.02
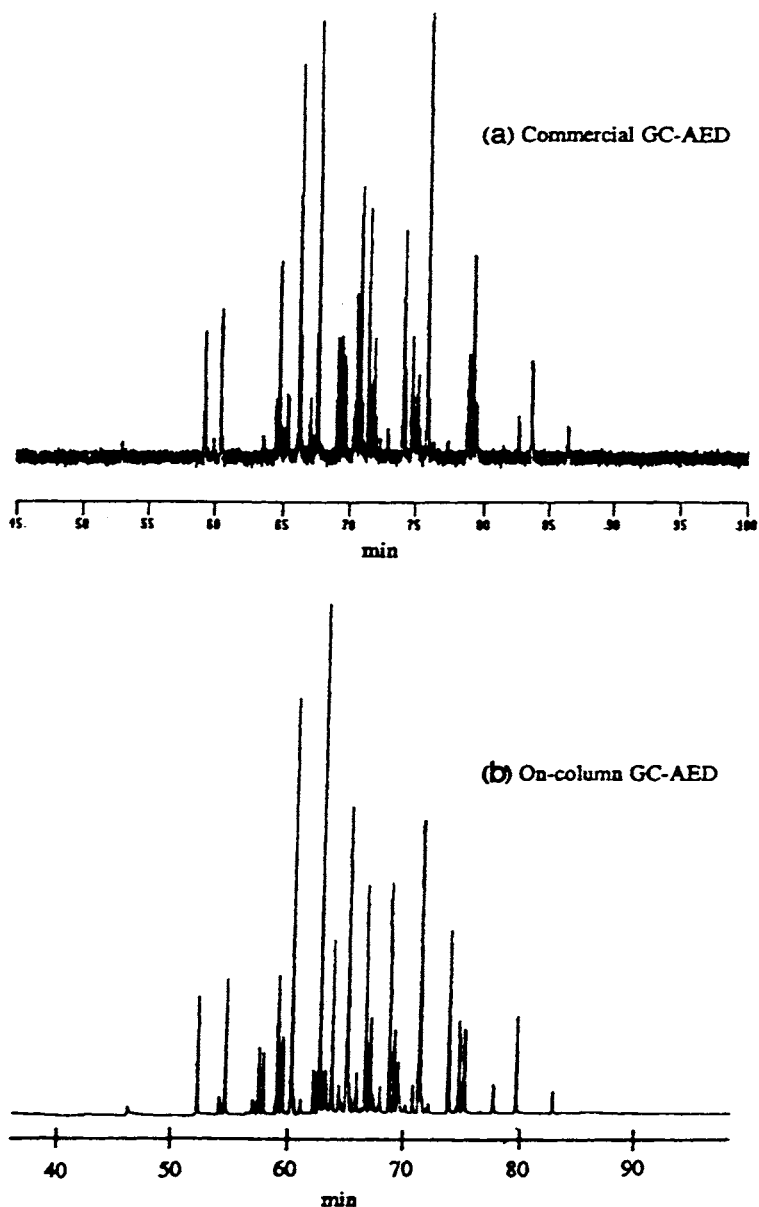
Fig. 5.11. Comparison of PCB detectability with (a) a commercial GC-AED equipment and (b) a laboratory-built on-column GC-AED system.

compared [34,35]. The sensitivity obtained by ECD was still the best with GC-AED being slightly higher than GC-MS.

Optimization of different parameters affecting the performance of AED for the enhancement of its sensitivity was quite important. The GC-AED sensitivity for brominated and chlorinated compounds was dramatically improved by further modifications and eliminat-
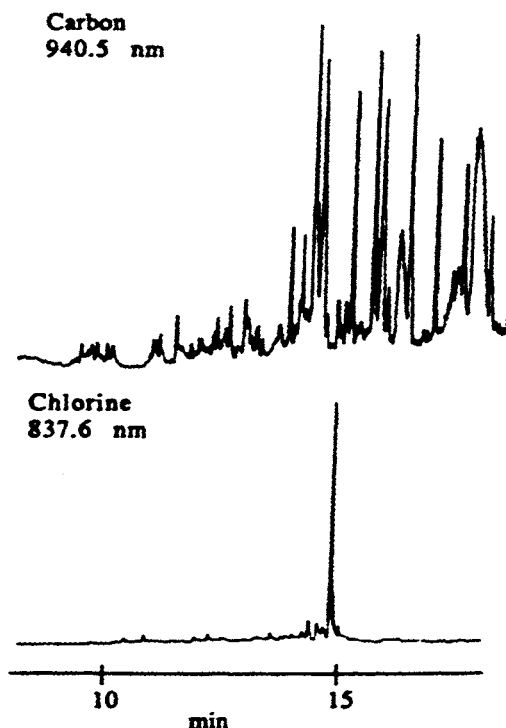
Fig. 5.12. C and Cl chromatograms of an extract of snow from Antarctica.

ing the introduction of make-up gas and sustaining the plasma inside the end of a fused-silica GC capillary column, so called on-column detection [36]. Fig. 5.12 shows the carbon and chlorine traces of a snow sample collected in Antarctica. On the carbon channel, the system performed as a universal detector, demonstrating the complex nature of the sample. The chlorine channel showed the presence of a single chlorinated compound. A commercial system was used for confirmation as well. The chemical structure of this compound was not recognized.

5.3.2.3 Pesticides

Pesticides are widely used in agriculture and for many other purposes around the world with more than 10 000 commercial formulations of approximately 450 pesticides currently in use [37]. Their extensive employment and dispersion in the environment has mainly occurred in the last decades. They are regarded as relatively ubiquitous pollutants in human and animal tissues, soils and crops and water. However, transfer to the atmosphere, the oceans and oceanic food chains has led to their wider global spread. Pesticide analysis, therefore, has become an important issue, as they are present in water, soil, food and foodstuffs.

A prototype of the HP 5921 A atomic emission detector in conjunction with a GC was used for the detection and elemental characterization of 27 different pesticides by obtain-

ing element-specific chromatograms for C, H, N, O, Br, Cl, F, P and S [38]. Applying quantitative analysis for each element, it was possible to calculate the approximate empirical formulas for 20 different herbicides in two different mixtures. At the end, an extract from an apple spiked with chlorpyrifos, endosulfan I and endosulfan II was analyzed by the GC-AED system.

Lee and Wylie [39] incorporated a multi-channel diode array detection system to monitor chlorine, fluorine and phosphorus-containing compounds in a green onion extract spiked with a number of pesticides at the sub-ppm level. In this work, the performance of AED in comparison with other specific detection methods for the analysis of pesticide residues in plant matrices was evaluated. The determination of phosphorus, sulfur and chlorine-containing pesticides with limits of detection ranging from 3 to 60 pg/s was demonstrated using a Surfatron-MIP, which operates by surface microwave propagation along a plasma column [40]. The effects of pressure, ranging from 20 to 760 Torr, were also examined and the best values were concluded to be found at 50 Torr.

A GC-MIP-AED method for pesticide residue determination in fruits and vegetables was reported by Ting et al. [41]. Six different aspects of research were studied: (i) sensitivity and linearity studies for elements S, P, Cl, and N by analyzing dursban; (ii) a study of instrumental response to Cl concentration in pesticide molecules; (iii) organochlorated pesticide recoveries; (iv) organophosphate pesticide recoveries; (v) carbamate pesticide recoveries; and (vi) investigation of metal-containing pesticides with plictran and vendex as standards were carried out. They found the order according to sensitivity and linearity to be as follows: S-181 > P-178 > Cl-479 > N-174. Instrumental response to the concentration of chlorine atoms in the pesticide molecule was linear, with a correlation coefficient of 0.89. Recoveries of organochlorinated pesticides were 91.7–109.3%, with the exception of citrus, where recovery was influenced by co-eluting interferences. They concluded that organophosphate recoveries were 73.2% or more and the values for carbamates were rather inconsistent.

As elemental responses of AED are compound independent, the possibility of calibrating the instrument with a single randomly selected reference compound prior to a universal calibration gained some attention. Some workers [42–44] have performed the practical aspects of this type of detection. The elemental responses for carbon, hydrogen, chlorine and sulfur were obtained based on a single reference compound. This type of calibration improved the qualitative data from the GC-AED and/or GC-MS analysis and was utilized to select a number of reference compounds with elemental compositions close to those of the analytes of interest prior to the quantitative analysis. The volatility and the chemical structure of the reference must be taken into consideration while the split/splitless capillary injection systems are used. The practical utility of universal calibration was illustrated by the determination of pesticides, i.e. lindane, heptachlor, aldrin and lindane, in stomach oil deposits from birds living in Antarctica.

The main drawback of AED is its lower sensitivity with respect to other selective detection methods regularly used in pesticide residue GC analysis, such as the ECD and nitrogen phosphorus detectors. As has been demonstrated earlier, the higher detection limits of AED have not been an obstacle for some researchers to employ this interesting and quite selective instrument. The developments of LC-GC instrumentation and the subsequent introduction of new methods for injecting large volumes of sample onto the

GC seem to be capable of compensating for this problem. A much better sensitivity can, therefore, be achieved, as it would be possible to inject as much as 100 μl of sample.

In an attempt to perform GC-AED for pesticide residue analysis in plant foodstuffs, Stan et al. [45] achieved 0.01 ppm concentration levels by an automated large volume injection with a programmed-temperature vaporizer (PTV) and solvent venting as well as careful optimization of make-up and reagent gases with AED. After removing lipids and waxes by gel permeation chromatography, extracts from 10 g of the food samples were concentrated to 200 μl, of which 12.5 μl was injected into the GC-AED system. Large volume injection was applied using a 5 m × 0.32 mm i.d. retention gap deactivated with phenylsilicone. They performed two analyses, recording the sulfur, phosphorus, nitrogen and carbon traces in the first run and chlorine and bromine traces in the second run. The limits of detection for 385 pesticides were determined. Studies on dependence of chlorine-selective detection on make-up gas flow and also oxygen gas flow are summarized in Fig. 5.13. A flow of 40 and 10 ml/min for the make-up and oxygen gas flow appeared to be the optimized values.

Due to the growing use of atomic emission as a detection technique, the potential of GC-AED for pesticide multiresidue analysis was explored by Bernal et al. [46]. Making a comparison study with data obtained by MS, retention data of 181 phytochemicals of diverse properties and considerations about the identification reliability and sensitivity were provided. The GC-AED system was applied to the target analysis of 11 herbicides in spiked soil samples. Complementary use of MS was shown to be necessary to resolve some peak pairs quite close to each other in AED. Trifluralin and chlorotoluron, were identified in a soil sample by GC-AED. Triallate, a herbicide recommended for chemical weed control in cereal, legume and beet crops, was extracted from soil by supercritical CO_2, followed by GC-AED analysis [47]. The selective extraction procedure, in conjunction with the selectivity of the sulfur channel of AED, provided a valid tool for analyzing this pesticide in soil at the trace level.

5.3.2.3.1 Water analysis   Monitoring of trace amounts of any organic analytes with polar moiety including the pesticides in aqueous matrices has gained a high degree of importance. However, in order to determine concentrations in low μg/l or even lower ranges, preconcentration of relatively large volumes of sample is often essential. In principle, trace enrichment can be performed by liquid–liquid extraction (LLE) and solid phase extraction (SPE). LLE tends to be rather laborious and time consuming, and its repeatability is not always satisfactory. SPE offers higher extraction efficiency, more selectivity and higher precision than LLE. The use of on-line SPE techniques is a good alternative and many well-documented applications have been reported. Some researchers have, therefore, tried to employ SPE to enhance the enrichment factor for organic pollutants and combine this highly efficient extraction method with GC-AED.

The use of a GC-AED system following a process of preconcentration of drinking water samples by a factor of 1500:1 allowed the highly selective determination of chlorophenols present in samples below the maximum limit of 0.5 ng/ml [48]. The preconcentration of the samples was carried out using 0.25-g commercial graphitized carbon cartridges without the need for sample derivatization prior to SPE.

In another attempt, solid phase microextraction (SPME) was shown to be an efficient way to isolate organophosphorus pesticides in water samples [49]. A fused-silica fiber
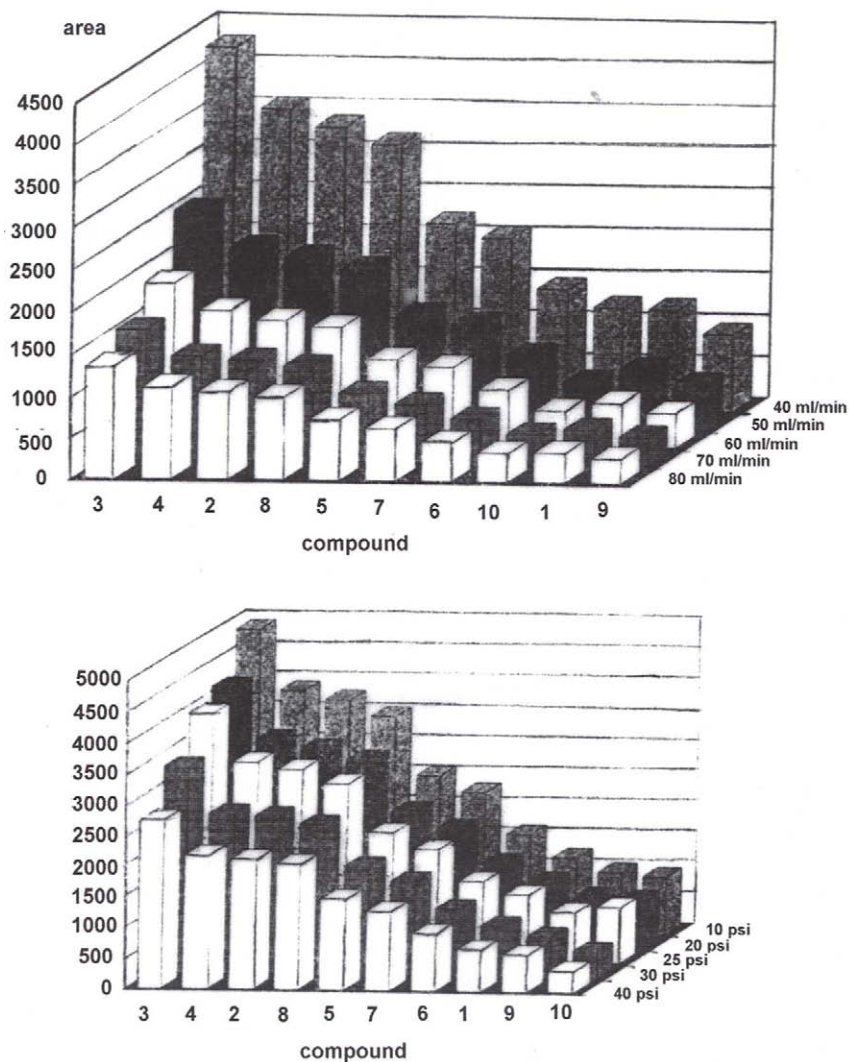
Fig. 5.13. Dependence of chlorine selective detection on (a) make-up gas flow and (b) oxygen gas flow.

coated with polydimethylsiloxane was employed to extract organic compounds and transfer them into the GC injector for thermal desorption and subsequent analysis. Volatile compounds such as organophosphorus pesticides could be efficiently isolated from aqueous samples. This method could afford limits of detection of ppb and sub-ppb level and showed a precision of 8–12% (RSD), depending on the compound. The described method only suits volatile species and suffers from the lack of accurate quantitative data. Lately, the use of automated SPME in conjunction with GC-AED for the determination of metazachlor in waste water was reported [50]. The method was based on diluting the
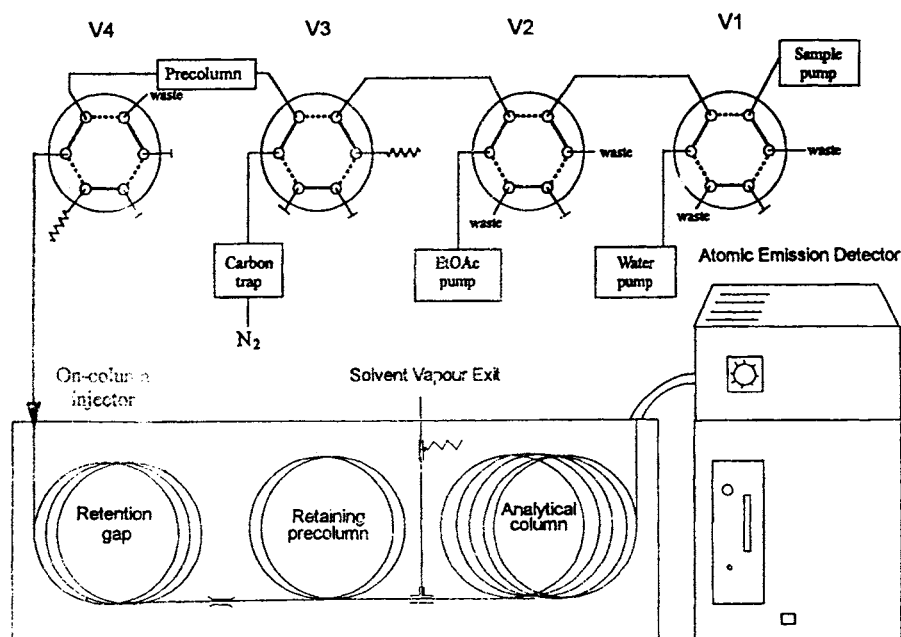
Fig. 5.14. On-line SPE-GC-AED system.

aqueous sample with a 10% NaCl solution to minimize the influence of matrix effects. The entire analysis was automated except for the initial addition of NaCl solution.

Goosens et al. [51] employed continuous LLE combined with on-line capillary GC-AED for water analysis. Only 10 ml of spiked water was injected into a water stream, with a flow of 1 ml/min, which merged with an iso-octane stream, having the same flow rate, via a segmentor and passed through a PTFE extraction coil to a phase separator. A 100-μl portion of the organic phase was injected into a GC-AED equipped with a large volume injection system including a 9 m × 0.53 mm i.d. fused silica retention gap and a 3 m × 0.31 mm i.d. precolumn coated with DB-17 stationary phase. Detection limits of 40, 10, 125 and 70 pg for N, S, C and Cl were obtained, respectively. Analyte recoveries were in the range 80–90% with RSD of 4%.

A set up for an on-line solid-phase extraction (SPE) in conjunction with a GC-AED system using an on-column interface to transfer 100 μl of desorbing solvent to the GC part of the system was developed [52]. A schematic diagram of the set-up of the SPE-GC-AED is shown in Fig. 5.14. The SPE module consisted of a valve switching unit with four pneumatic six-port valves. A 80 cm × 50 μm i.d. restriction capillary was installed between the pump and valve V2 to provide a constant and pulse-free flow of 76 μl/min. For the analysis of aqueous samples a 2.2-ml loop mounted on valve V1 was used, and then was flushed into the precolumn with 4 ml of HPLC-grade water. The SPE unit was interfaced to the GC system via a 30 cm × 50 μm i.d. fused silica capillary. This capillary which was permanently located in the on-column injector, was inserted into a 5 m × 0.32 mm i.d. retention gap. A press-fit connector was used for connecting the retention gap to a 3 m × 0.32 mm i.d. retaining precolumn. The analytes were desorbed and transferred to the

TABLE 5.4

COMPARISON OF DETECTION LIMITS (ng/l, S/N = 3) FOR OPPS IN AQUEOUS SAMPLES USING OFF-LINE AND ON-LINE SPE-GC-AED

Compounds	Detection limits (ng/l)		
	Off-line with	On-line with	
	10 ml, 1 μg/l sample	10 ml, 0.1 μg/l sample	100 ml, 10 ng/l sample
Mevinphos	50	–	–
Sulfotep	15	5	0.5
Diazinon	30	10	1.0
Fenchlorphos	30	10	1.0
Parathion	30	15	1.0
Bromophos-ethyl	150	10	1.0
Tetrachlorvinphos	70	30	1.5
Ethion	40	5	0.5
Triazophos	75	20	1.5
Pyrazophos	130	15	1.0
Coumaphos	275	30	1.5

GC separation column using 108 μl ethyl acetate. An early solvent vapor exit (SVE) was inserted between the retaining precolumn and the analytical column to create the desired evaporation rate. The oven temperature during analyte transfer was 80°C and after 5 min the temperature was raised to 280°C at 20°C/min and finally held at 280°C for 2 min. Table 5.4 compares detection limits (ng/l, S/N = 3) for organophosphorus pesticides (OPPs) in aqueous samples using off-line and on-line SPE-GC-AED. In this work, analytical characteristics such as recovery, precision and linearity of calibration curves were comparable with those of the off-line combination of SPE-GC-AED using OPPs as test compounds. This set-up was employed for the analysis of municipal waste water; 10 ml waste water samples were analyzed. The samples were taken before and after biological treatment. Typical chromatograms for both influents and effluents recorded on carbon (A and B), nitrogen (C and D), and phosphorus (E and F) channels are shown in Fig. 5.15. Since the sampling from both sites was done simultaneously, no definitive conclusion can be drawn from the several traces; the general ''clean-up'' of the treatment procedure is obvious from the carbon and nitrogen traces, but the phosphorus traces looked different. The fully on-line set-up caused a marked enhancement in sensitivity because of the quantitative transfer of the analytes from the SPE module to the GC. Detection limits were reported as 5–20 ng/l for the analysis of 10-ml raw and spiked surface water samples when monitoring the phosphorus channel.

Although many great separation successes have been achieved so far, and in many cases difficult components can be separated from each other, in real-life situations there are still many co-eluting compounds for which even partial separation would take great efforts. The employment of MS detection for chromatographic methods certainly has increased the selectivity but for some isomers and the co-eluted pairs, specifically for those having strongly different peak heights ratio, the problem of resolution and subsequent identification is not yet solved.
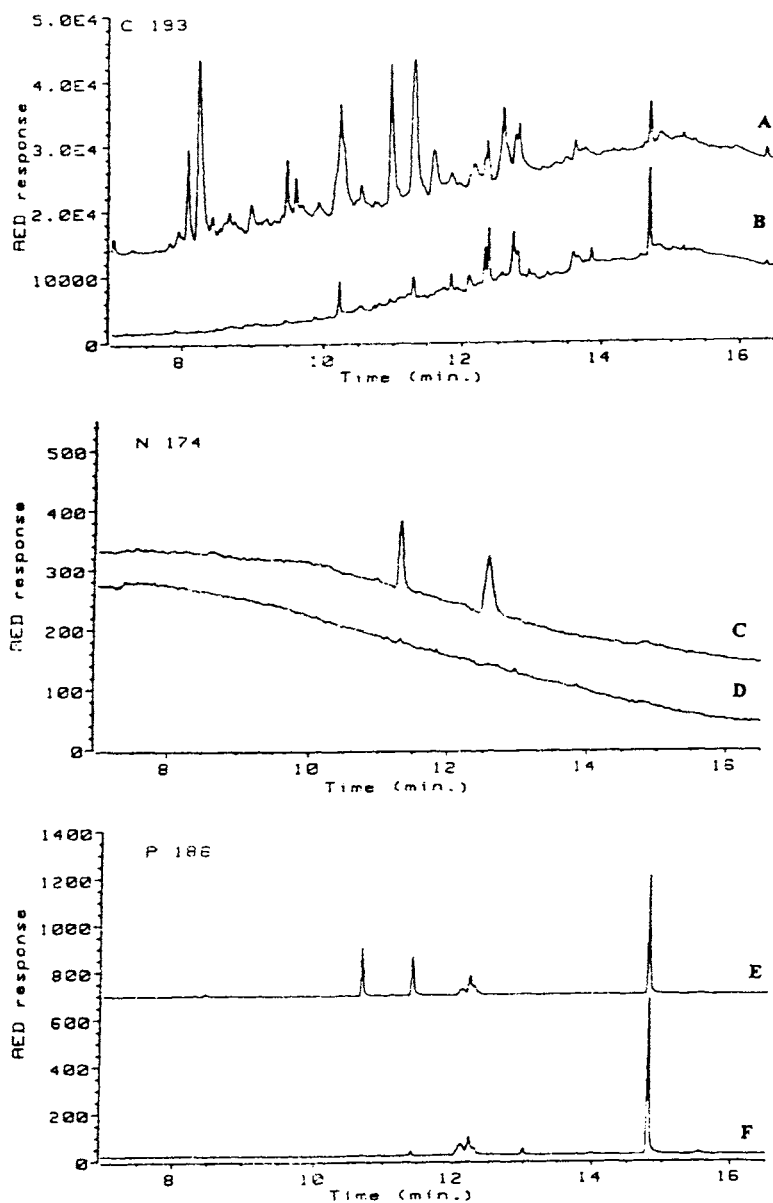
Fig. 5.15. On-line SPE-GC-AED of 10 ml waste water samples using (A,B) C-193, (C,D) N-174 and (E,F) P-186 channel. Influent (A,C,E) and effluent (B,D,F) analyzed.

Hankemeier et al. [53] described a procedure for the screening of heteroatom-containing compounds in tap and waste water by correlating data obtained by GC-AED-MS. They were able to focus attention on peaks representing compounds present in the samples at or above a predetermined threshold level of, typically, 0.1 or 1 μg/l. They, therefore, required the application of SPE-GC procedures to achieve analyte trace enrichment and, on a quite

different level, experimental verification of the validity of the universal calibration concept. A concept for the screening of relevant compounds by systematic and unambiguous AED-to-MS correlation of relevant peaks in water samples and subsequent quantification was developed and applied. The potential of the approach was shown by the identification of target compounds as well as all unknowns present in drinking and waste water above the predetermined threshold of 0.05 or 0.5 μg/l of drinking and waste water samples, respectively.

In a recent study, the coupling of an AED and MS detector to a single gas chromatograph was described by Mol et al. [54]. Organic microcontaminants were detected via their heteroatom AED traces, whereas parallel-acquired mass spectra allowed confirmation and identification. The possibility of AED elemental calibration was studied for vegetable and water samples. The AED and MS results were in good agreement with each other. The system was used for the non-target analysis of river water and a number of heteroatom-containing microcontaminants were detected, identified and quantified down to the 20 ng/l level.

More recently, the potential of a GC system with simultaneous AED and MS detection using a post-column split for trace analysis of co-eluting pollutants in surface water was examined [55]. The data from both detection systems were directly correlated with no problems occurring regarding retention time differences. Capability of the method for the identification and quantification of some co-eluting organic micropollutants in River Karoun (Iran) water was demonstrated. The applicability of the system towards the identification and determination of unknown pollutants in River Karoun water was examined. Table 5.5 lists the names, retention times, major ions, AED channel(s) and concentrations of some of the pollutants found in River Karoun water. The primary survey on the total ion chromatograms (TIC) led to the identification of a number of pollutants present in the river

TABLE 5.5

NAMES, RETENTION TIMES, MAJOR IONS, AED CHANNELS AND CONCENTRATIONS OF SOME POLLUTANTS IDENTIFIED IN RIVER KAROUN WATER BY THE SIMULTANEOUS GC-MS-AED SYSTEM

Compound	t_R (min)	Major ions (m/z)[a]	AED channel	Conc. (μg/l)
2,4-Dichloro-3,5-dimethyl phenol	9.35	155, 190[b], 192, 157, 91, 119	C, H, Cl	–
2,6-Di (*t*-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	9.76	165, 57, 180, 137, 205, 123, 221[b]	C, H	–
2,6-Di (*t*-butyl)-4-methyl phenol	9.99	205, 57, 191, 220[b]	C, H	–
Atrazine	11.42	200, 58, 92, 215[b], 68, 173	C, H, Cl	0.2
Diazinon	11.62	137, 179, 152, 199, 304[b], 93, 124, 65,	C, H, S	0.3
Ametryn	12.19	227[b], 212, 58, 170, 98, 185	C, H, Cl	0.2

[a] According to their relative intensities.

[b] Molecular ion peak.

water sample. However, consideration of the AED chromatograms, especially those recorded on sulfur and chlorine channels, provided strong evidence for the presence of some pollutants containing sulfur and chlorine atoms. The chlorine trace was recorded subsequently after the carbon, hydrogen and sulfur channels due to the limitation on the number of elements that could be recorded simultaneously in a single run. The retention times recorded on this chromatogram, therefore, show a very slight difference. Exact retention times of these compounds from the AED channels were used for screening of TIC to extract the spectrum of unknown compounds. In some instances the volume of water samples for the SPE was increased. This led to the appearance of stronger signals on TIC and therefore, the library search strategy was more feasible. The extracted ion chromatograms, at *m/z* values of 200, 304, and 227 and their corresponding mass spectra, along with the AED traces strongly indicated the presence of atrazine, diazinon and ametryn, respectively. The concentrations of the three pesticides identified in the River Karoun water were at the sub-ppb level. 2,4-Dichloro-3,5-dimethyl phenol, 2,6-di (t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one and 2,6-di (t-butyl)-4-methyl phenol were tentatively identified as chlorophenolic compounds and antioxidants, respectively. GC-MS-AED with the simultaneous detection facilities, in conjunction with SPE, showed to be a highly selective and sensitive method for the discrimination of some co-eluting components and the identification and determination of micro-organic pollutants. The two detection devices using a post-column split can generate up to five chromatograms, including the TIC trace and the data from both detection systems are directly correlated without retention time differences. The role of AED when heteroatom molecules are co-eluted becomes increasingly important.

5.3.2.4 Others

Environmental applications of GC-AED have been extended to many substances including polycyclic aromatic compounds (PAC), chlorophenols and semivolatile environmental compounds.

The pyrolysis of coal tar pitch produces volatile aromatic and polycyclic aromatic compounds. These gaseous products were analyzed by GC-MS and GC-AED [56]. After preconcentration of the sample on a XAD-2 sorbent and desorption of analytes by toluene, an aliquot of 0.5–1.0 μl was injected into the capillary GC. About 600 different compounds were identified in the flue gas by their GC retention times and their electron impact (EI) mass spectra recorded on GC-MS. The sulfur channel trace allowed 29 sulfur-containing PACs to be identified. Separation of a sulfur-containing fraction of coal pyrolysis products was also carried out by on-line LC-GC-AED multidimensional methods [57]. The use of on-line LC-GC-AED enabled the analysis of complex samples to be simplified. Benzothiophenes and C1–C5 substituted dibenzothiophenes were identified by monitoring the sulfur channel trace, recorded at 181 nm. The on-line fractionation procedure increased the sensitivity of the method with the removal of interfering compounds, mostly saturated and unsaturated hydrocarbons, allowing sample enrichment with minimum sample loss, contamination and oxidation.

Recently, in an attempt to apply GC-AED as a routine environmental screening tool, 58 typical environmental contaminants were analyzed by GC-AED and detection limits and elemental response factors (ERFs) were determined for hydrogen, nitrogen, oxygen, and

chlorine [58]. Regression analyses were carried out from the low nanogram to picogram detection limit to the upper loading limit of the GC column, i.e. 50 ng. Using the ERFs of standard compounds, the empirical formulae were determined for 19 chlorinated hydrocarbons and eight chlorinated sulfur-containing hydrocarbons in a complex environmental sample.

Rodriguez et al. [59] reported a method for the determination of chlorophenols in waters that involved direct acetylation in the sample using alkaline acetic anhydride, followed by solid-liquid extraction of the derivatives on graphitized carbon and detection by GC-MIP-AED. The method was applied to the analysis of natural and tap water samples. After preconcentration of one liter of unspiked tap water sample, 2,4,6-trichlorophenol was found to be present even though the intensity of the peak was not strong enough to reach the limit of quantification. Its concentration, therefore, was calculated to be below 0.09 ng/ml, although the authors did not mention any problem related to breakthrough volume.

5.4 CONCLUSION

GC-AED has been shown to be a highly selective and relatively sensitive technique and has been utilized in academic, governmental and industrial laboratories. Since its commercial introduction as an integrated system, the technique has been applied more extensively to trace analysis, a vital issue in environmental studies. For metal heteroatom species, derivatization of metal-containing species to a more volatile compound is necessary. This elemental-selective system is also well suited for the analysis of organic compounds and has been applied to most types of environmental samples. Responses for heteroatom-containing compounds are more or less independent of molecular structures and linear. These types of compounds may, therefore, be quantified based on universal calibration, where the peak areas for all the analytes of interest are related to a single heteroatom calibration curve obtained for a randomly selected reference.

Since MIP-AED has been developed as a selective detector for GC, all trace enrichment techniques amenable to GC, i.e. phase separator LLE, SPE, SPME and large volume injection methods have been coupled to this integrated system. With this approach, low ng/l detection limits for a variety of chemicals in different environmental samples have been obtained.

Next to GC-AED, the use of other analytical techniques, i.e. GC-MS and GC-FTIR, seems to be quite beneficial as they provide complementary data for the identification of close-eluting peaks.

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Chapter 6

Methods for the determination and evaluation of chlorinated biphenyls (CBs) in environmental matrices

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CONTENTS

6.1 INTRODUCTION

Polychlorinated biphenyls (PCBs)[1] have been manufactured in substantial amounts since the 1920s [2]. Their use in the electrical, paint, pigments, paper and cardboard industries and subsequent disposal into the environment during the intervening years allowed sufficient time for these compounds to spread to the remotest areas of the world before any control on use or disposals was implemented. From the initial detection of PCBs in biological tissue in the 1970s [3,4] by Jensen there has been a continuous development in the analytical techniques to determine these chemicals [5–9]. The chronological development of the key aspects of the analysis of chlorobiphenyls (CBs) from 1970 is given in Table 6.1.

The high hydrophobicity, lipid solubility and persistence of CBs has resulted in widespread contamination of biological tissue to the extent that all environmental compartments that have been analysed contain measurable levels of these contaminants [10–17]. The toxic nature of these compounds has required a series of extensive risk assessments to establish the threat to both man and to wildlife [18,19]. Between 1993 and 1999 there have been a number of general reviews on CBs as well as articles which cover more specific aspects of extraction, separation or measurement of the congeners. Reference to these articles and their subject area are given in Table 6.2.

The occurrence of PCBs as complex mixtures has a considerable impact on the costs of a complete analysis. Highly sophisticated techniques are required and clean-up methods are labour intensive. Multi-residue methods [20,21], in which several polyhalogenated hydrocarbons (PHHs) groups can be determined in parallel are available, but this approach is often impractical without some compromise resulting in lower recoveries and unresolved peaks in the chromatograms.

Recent developments in the determination of CBs has been driven by the need to improve the reliability of the data in a cost effective way. Sample preparation using conventional Soxhlet/blending/ultrasonic extractions and gravity adsorption columns for clean-up is labour intensive, prone to contamination from the laboratory atmosphere or from excessive handling. The focus in recent years has been to miniaturise, reduce solvent volumes and containerise the sample. In this way the individual extraction and clean-up modules can be automated into an integral analytical system. The continued development of software controlled sample preparation, separation techniques and the final determination has improved the reliability of the data in many laboratories. This has allowed a more detailed interpretation of the CB data, including the *toxic* CBs. As a result reliable patterns of congeners in different environmental compartments have been identified using a wide range of multivariate techniques to provide a more informed evaluation of these contaminants.

The specific areas of development in CB analysis over the past 5 years are:

- automation with microwave assisted extraction (MAE) and accelerated solvent extraction (ASE);
- supercritical fluid extraction (SFE) with modifiers and solid phase traps for simultaneous clean up;

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[1] The term polychlorinated biphenyl (PCB) refers to the technical mixtures found in the formulation and the measurements made on the basis of calibration with these mixtures. Chlorobiphenyl (CB) refers to the individual congener named by the Ballschmiter number [1].

TABLE 6.1

CHRONOLOGICAL DEVELOPMENT OF CHLOROBIPHENYL ANALYSIS

Year	Development	Reference
1925	Large scale manufacture of PCB formulations	
1966	First reported measurement of PCBs as 'Avian Peaks'. Packed GC column separation of PCBs. Concentration in samples estimated against industrial formulations and summation of mixed component peaks in the chromatogram	[139,229]
1969	Development of adsorption column chromatography for clean-up of biological tissue	[230]
1975	Introduction of glass capillary columns. Improved separation of PCBs	[140]
1970s	Development of stable GC ovens and electronics to improve reproducibility of retention indices	
1980	Individual chlorobiphenyl congeners identified and systematically numbered. Introduction of fused silica capillary columns. Improved column stability	[1]
1984	Retention times of all 209 CBs measured on a SE 54 capillary column	[146]
1985	Reference materials, certified for individual CBs, become available. Commercial availability of many of the 209 CBs. Reports of retention times on polar and semi-polar stationary phases. Development of multi-dimensional chromatography	[231,232]
1988	Focus on the analysis of planar, toxic CBs and the application of toxic equivalence concentrations (TECs) for CBs as well as dioxins	[233]
1989	Separation of CBs on the basis of their spatial configuration. Identification of all congeners present in main commercial formulations	[126,127]
1989–1993	LC/GC online coupled	[129,135,236]
1990	Use of pyrenyl-silica HPLC for separation of non- and mono-*ortho* CBs	[127]
1990s	Development and application of novel extraction techniques including accelerated solvent extraction, microwave assisted extraction, Soxhtec, supercritical fluid extraction	[235]
1992	Expansion of retention data for five GC phases of different polarity for all congeners in commercial mixtures (>0.05%), except CB69, 75, 96 and 182: series coupled columns	[142,148]
1993	SFE-GC coupled techniques	[79,237]
1995	Improvements of pyrenyl-silica HPLC through temperature control	[128]
1994/8	Use of pyrenyl-silica column for separation of CBs and PCDD/Fs	[41,234]
1997/9	Modular multidimensional gas chromatography	[156,158]

- individual CB analysis with multidimensional gas chromatography (MDGC) including the analysis of enantiomers.
- modulated MDGC;
- atomic emission detectors (AED), diode array detectors (DAD) and enzyme-linked immunosorbent assay (ELISA) as alternative *detectors* to the electron capture detector (ECD) and the mas spectrometer (MS);
- quality assurance (QA) programmes including laboratory performance studies and improvement programmes;
- data analysis for monitoring, legislation and research;
- health and safety at work (non-chlorinated solvents).

This chapter reviews the developments for the determination of individual CBs since the first edition of this book in 1993 [22] and includes the non-*ortho* chloro and mono-*ortho* chlorobiphenyls. Information relating to the different aspects of the methodology have been tabulated for comparison with the earlier key developments, leaving the more detailed comments on recent studies. An overview of recent applications of the analysis of CBs in environmental matrices is given in Table 6.3.

6.2 SOURCES OF PCBs

The production, properties and usage of PCBs have been extensively reviewed [23–25]. There are no natural sources of PCBs and these contaminants are present in the environment from accidental or deliberate release, or permitted discharge from direct manufacture from use in either open systems such as additives to plastics or in closed, sealed systems like transformers. PCBs are no longer used in open systems in most developed countries e.g. as additives in paints. Manufacture and new applications have now been banned or severely curtailed. However, while the production of most chlorinated hydrocarbons have been terminated in most countries since the 1980s, the former USSR were still manufacturing and using PCBs well into the 1990s [26]. PCBs also contain impurities in manufacture and subsequent use of the primary product which may also be both toxic to the environment and may also interfere with the analysis of the CBs. Polychlorinated naphthalenes (PCNs) are present as impurities in PCB manufacture. In addition, Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzo furans (PCDFs) are present as impurities in the high temperature chlorination manufacture, but are more likely to be formed through the disposal of PCB impregnated materials. PCDDs and PCDFs are formed in the incineration of such material containing PCBs, PCNs and polyvinylchloride.

Secondary sources of PCBs come from leachates from land fill sites, incineration and combustion, waste water discharges and sewage sludge disposal on land or at sea. Not only are most of these compounds stable and persistent, but the sources provide a relatively continuous input into the wider environment. Cessation of manufacture or the use in closed systems may have reduced the volume of the source, but with the mass of these PCBs in land fill sites and in goods in homes and industry there is a *virtual low level leak* into the wider environment. It is inevitable that most of the primary production of these PCBs will find its way onto the land or into the rivers and the sea. Recycling through vaporisation and aerial transport only serves to dissipate these compounds to the more remote areas of the world. These compounds enter the food chain via uptake on the land by animals eating the vegeta-

TABLE 6.2

REVIEW ARTICLES ON CB ANALYSIS

Review area	Reference
Review of CB analysis	[22]
Review of non-*ortho* CBs	[57]
Review of environmental analysis of chlorinated thioethers, sulphoxides and sulphones	[238]
Determination of chlorobiphenyls in sediment	[92]
Review of CB analysis	[239]
Review of extraction of CBs from seawater	[56]
Thesis on analysis and biomonitoring of complex mixtures of persistent halogenated contaminants	[179]
Thesis on planar aromatic compounds – PAHs, CBs, PCDD/Fs	[41]
Polyhalogenated hydrocarbons in food, including CBs	[240]
Modulated multidimensional GC	[156]
Comprehensive book on PCBs	[25]
Thesis on semi-permeable membrane devices (SPMD)	[241]
General review on immunoassay techniques	[242]
Enzyme-linked immunosorbent assay (ELISA)	[84]
Microwave assisted extraction (MAE)	[84]
Supercritical fluid extraction (SFE) in environmental analysis	[243]
Analytical scale SFE for environmental contaminants	[244]
Environmental applications of SFE including CBs	[245]
Capillary GC coupled with atomic emission detector (AED) for environmental samples	[185]
Enantiomer determination of chiral organochlorines in biota by GC on modified cyclodextrins	[161]

tion or animal feed contaminated at low levels or similarly through the aquatic food web to fish, shellfish or for some specific communities, to marine mammals.

The realisation that these secondary sources, in particular, will be present for some years to come places a requirement upon the analyst to provide a fast, cost effective method which provides an unequivocal measurement of the target congeners.

6.3 COMPOUND SELECTION

The first separation of PCBs was obtained using packed gas chromatographic columns with industrial formulations as calibration standards to quantify a single total value for the PCB [25]. This early technology did not have the resolution to separate the PCBs in the environmental samples or formulations into individual CBs and the most appropriate method to estimate these contaminants at that time was unquestionably by the summation of the peak heights or areas of the low resolution chromatogram. Some workers recognised the potential errors in such estimates and attempted to obtain a single response by perchlorination to the decachlorobiphenyl (CB209) [6,27,28]. The need to improve the separation, identification and quantification of the individual CBs has been reinforced by measurement

of the toxic, biological effects of specific congeners [18,19,29,30]. Over the past 10 years analytical developments have centred on the determination on the non-*ortho* chloro and mono-*ortho* chlorobiphenyls [18,19] which are known to be toxic and induce liver microsomal enzyme activity. These CBs induce both aryl hydrocarbon hydrolase (AHH) and ethoxy resorufin-*o*-deethylase (EROD), bind with high affinity to the cytosolic receptor protein and are iso-electronic with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Both the toxic mechanism and the enzyme induction involve an initial binding of the CB to the same aryl hydrocarbon, Ah, receptor.

The developments in analytical methodology for the measurement of CBs has progressed in response to the purposes of the analysis. It is essential that the measurements remain *fit for purpose* and fulfil the primary reasons for obtaining them. These include:

- national and international monitoring for spatial distributions or temporal trends [32–34];
- legislative requirements in relation to leachates and discharges [35–37];
- in support of biological effects monitoring and research [38,39];
- provision of toxic equivalences (TEQs)[2] legislative or biological effects correlations [40];
- patterns within and between species and environmental compartments [41,42].

Of the 209 CBs, 132 have been measured in industrial formulations at or above the 0.05% level [7]. The original selection of CBs, made by the European Union Community Bureau of Reference in 1982, was based on persistence, relative abundance, toxicity, and the unequivocal measurement of the individual CB in a specific matrix [43]. This selection included CB28, 52, 101, 118, 138, 153 and 180 and has now been adopted widely in many terrestrial, marine and food monitoring programmes [32,44,45]. However the limited number of congeners was insufficient to study specific toxicological effects and spatial patterns of these contaminants. These congeners, on their own, do not provide data for the TEQ values needed for legislative or for environmental policy purposes.

Studies on the measurement of the toxic, planar CBs has been given added impetus by the correlation of EROD measurements with the determination of the toxic equivalent concentrations (TEC) [12,18,19,40].

The main groups of CBs are:

- non-*ortho* CBs: 77, 81, 126, 169;
- mono-*ortho* CBs (penta): 105, 114, 118, 123;
- mono-*ortho* CBs (hexa): 156, 157, 167;
- mono-*ortho* CBs (hepta): 167.

The toxic equivalency factors (TEFs) for these CBs have now been agreed and are currently standardised [40]. In many respects the measurement of CBs has reverted to the summation of a single value. Previously this value was simply the sum of unresolved congeners where the present TEQ is the total of the toxic congeners expressed as TCDD. Most of the raw CB data produced by competent laboratories with a quality management system will provide information on the precision of these measurements. The calculation of

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[2] TEQs refer to the sum of the concentration of the congener equivalent to 2,3,7,8-TCDD obtained by using the toxic equivalence factor.

TABLE 6.3

APPLICATIONS OF THE ANALYSIS OF CBs IN ENVIRONMENTAL MATRICES[a]

Matrix	Reference – non-planar CBs	Reference – planar CBs (including mono-*ortho* and non-*ortho* CBs)	References – CB enantiomer
Human adipose tissue	[136,160]	[160]	
Invertebrates	[246]		
Formulations		[247]	[170]
Fish and shellfish	[160,203,248,249]	[48,50,160,190,250]	[169]
Fish and shellfish RM	[91]		
Incineration/fly ash		[251]	
Terrestrial mammals	[252]	[173]	
Marine mammals	[152,203,253,254]		
Sediment	[74,86,92,187,195,196,203,246,255–259]	[41,255]	[170]
Sediment RMs	[74,75,81,85,91,195]		
Sewage sludge + RMs	[76,257]		
Soil	[81,84,195,196,256,260]	[247]	
Soil RMs	[91,195,261]	[131]	
Water	[255,256]	[255]	
Interlaboratory studies	[203,207,209]	[262]	
Methodological development	[80,84,85,92,91,149,172,186,196,208,258,263]	[128,130,132,149,208]	
Multi-residue methods	[106,257]		
Review	[19,161,239,243,264]		

[a] Summary of applications since the previous edition of this chapter [174].

the TEQ values involves the normalisation of each congener with its own TEF value, but the final additive result is rarely reported with an estimate of the uncertainty for either the analytical variability or the TEF values. In most cases the overall uncertainty of the TEQ is large [46].

The main analytical difficulties are still the separation of these congeners (i) from other co-extractants both at the bulk level, e.g. lipids and at the trace contaminant level e.g. chlorobornanes (Toxaphene) and (ii) from other interfering congeners e.g., CB77 and CB110.

With the present technology and available methodology [41,47–49] it is now possible to measure individual CBs routinely at the pg/kg, and with care at the fg/kg.

The lower limits of detection can be extended by starting with a larger sample mass. However, additional clean-up steps are usually required to eliminate the increased mass of the co-extractants. The lower final volume of the sample extract also has a finite limit. This volume has typically been ca 1 ml. The introduction of micro-vials has allowed this volume to be reduced to ca. 50–100 μl, with an increase in sensitivity of ×10–20. Although further reduction in the solvent volume is possible, the impurities in the solvent are also concentrated ×100–1000. So there is little to be gained in additional solvent reduction for quantitative analysis. Manual manipulations at low volumes are not only difficult to manage quantitatively, but also incur the likelihood of contaminating the sample. Reduction in the detection limit is essential for some studies. Research on the metabolic pathways and pattern recognition of CBs has made it necessary to *measure* a wide range of congeners (20–50 CBs) in each sample. Since most methods of multivariate analysis require a complete data set, *less than* values, below the reporting limits, are of very limited value in such programmes.

Most of the analytical methods for non-*ortho* CBs are comparable to those used for *ortho* CBs. However, non-*ortho* CBs can occur at ca. 1000-fold lower concentrations (ng/kg range) than *ortho*-PCBs (μg/kg range) [50] and it is difficult to measure these compounds simultaneously with the *ortho*-CBs. An additional fractionation step is usually included in the clean-up procedure and a mass spectrometer (MS) is used instead of the electron capture detector (ECD), which enables isotope-labelled (^{13}C) standards to provide a measurement less prone to interferences and to compensate for recoveries of <100%. The non-*ortho* CBs are usually determined in a separate fraction, along with the PCDDs and PCDFs, because a greater sample mass is required to provide the necessary sensitivity. A schematic diagram of the isolation, separation, clean-up, group separation and final detection for CBs in the environmental matrices is given in Fig. 6.1.

6.4 MATRIX EXTRACTION

The method of extraction of CBs from environmental samples is dependent on the phase of the matrix. The isolation of CBs from air has primarily been based on solid phase extraction by absorption onto an organophilic substrate such as Tenax, followed by desorption and cryofocusing within the GC. Most airborne CBs are adsorbed onto particulates and so that the *extraction* is effectively one of filtering. The extreme case of this type of sampling is that of fly ash associated with incineration [51].

Isolation of CBs from the aqueous phase has focussed on three main techniques:

Water | Semi-Permeable Membrane Devices | Dialysis | Pyrene silica | MDGC in Series or Parallel | Mass Spectrometric Detector

Milk, oil, fat Mineral Oil | Dissolved Liquid - Liquid Extraction | Gel Permeation Chromatography | Porous Graphitic Carbon | Modulated MDGC | Electron Capture Detector

Blood | Solid Phase Extraction | Sulphuric acid - Silica gel | Charcoal/ Carbon fibre | HRGC eg HT-5

Plant material | Accelerated Solvent Extraction | Adsorption Chromatography Florisil, Alumina Silica | Planar phase Modified Silica | Chiral Phase Separation | Atomic Emission Detector

Sediment Sludge, Soil | Microwave Assisted Extraction | Saponification | | |

Animal tissue | Supercritical Fluid Extraction | Sulphur removal | | LC-GC |

| | Solid Phase Extraction | | | Diode Array Detector

Fly Ash | Column extraction Blending, Soxhlet | | | |

| Saponification | | | | ELISA

Air, Flue Gases | Ultrasonic | | | |

Fig. 6.1. The schematic flow diagram of the analysis of chlorobiphenyls. The connecting routes show the types of environmental samples, the methods of extraction which are currently available, the clean-up techniques, the group separation, high resolution chromatographic separation and the range of detectors used for the final measurement.

- liquid–liquid extraction (LLE);
- solid phase extraction (SPE);
- semipermeable membrane devices (SPMDs).

Most of the recent developments in extraction techniques have built on the methods developed earlier of Soxhlet extraction, blending and/or ultra sonication and column percolation. These methods have been used with a variety of solvent combinations and additives (Table 6.3). Extraction of organic contaminants from biological tissue and soil/sediment is also covered elsewhere [52]. Most methods have been based on common principles:

- polar and apolar mixed solvents are usually required for maximum extraction efficiency;
- extraction is kinetically as well as thermodynamically dependent;
- high purity solvents (>99.99%) are essential;
- the cleanliness of the laboratory is also essential. The condition of the laboratory is often the limiting factor in the analysis of low levels of CBs. Atmospheric contamination may be checked regularly with C_{18} discs by exposure for specific time periods and then analysing the discs for the presence of CBs (or other organic contaminants, e.g. phthalates).

TABLE 6.4

METHODS OF EXTRACTION OF CBs

Matrix	Method	Conditions/comment	Reference
Seawater	Methods, detection, recovery and validation	Review	[56]
	Solvent extraction LLE[a], polyurethane foam and amberlite	Comparison of LLE and SPE[a] extraction	[265]
	PEFS[a],LLE of dissolved phase with DCM	Separation of particulate and dissolved CBs	[266]
	Counter current flow extraction	Overcomes the problems of colloidal and particulate phases	[267]
	LSE[a]	XAD-2 and XAD-4	[268–270]
	LSE	C_8 and C_{18} columns	[271]
Blood and milk	Comparison of SFE[a] and LLE	florisil/CO_2, hexane (whole blood), cyclohexane-DCM[a]	[272]
Blood	Polytron homogenizer	EtOH–hexane–$(NH_4)_2SO_4$ saturated H_2O (1:2:1)	[273]
Crab tissue	Ultrasonic disintegration	H_2O (15 ml):macerated fat (10–20 g):acetone (30 ml):cyclohexane (40 ml)	[274,275]
Polar bear tissue	Ultrasonic disintegration	H_2O:macerated fat (10:1), acetone:hexane (3:2)	[275,276]
Fish and animal tissue	LSE in separating funnel	DCM–hexane (1:1)	[8,94]
Fish tissue	Column percolation	Grind Na_2SO_4 and Sio_2, soak 30 min, drain, repeat	[68]
Seal tissue	Ultra-turrax	3×40 ml DCM–MeOH (2:1) for 8 g sample	[95]
Soils and sediments	Microwave-assisted extraction	Hexane:acetone (1:1) (30 ml), 115°C, 10 min, 1000 W	[84]
Sand, soils and air filters	Microwave-assisted extraction	Hexane: acetone (1:1) (30 ml) 115°C, 10 min, 1000 W	[85]
Soils	Microwave-assisted extraction	1 ml iso-octane, 9 ml hexane:acetone (1:1) 1000 W, 15 min, 115°C	[277]
Soil (ERA-CRM) and sediments (NRCC HS-1 and HS-2)	Microwave-assisted extraction	30 ml hexane:acetone (1:1), 1000 W, 115°C, 10 min	[195]
Sediments	Microwave-assisted extraction	10 ml toluene:water (10:1), 66 W, 6 min	[86]
Sediments, urban dust, fish tissue (SRMs)	Pressurized fluid extraction and Soxhlet. Comparison of techniques and solvent	100°C 5 min, static 5 min, 2000 psi, DCM, acetonitrile, hexane:acetone (1:1)	[278]

[a] LLE, liquid-liquid extraction; SPE, solid phase extraction; DCM, dichloromethane; SFE, supercritical fluid extraction; PEFS, pressurised extraction and filtration system; LSE, liquid solid extraction.

The methods used to extract CBs from sediments, soils and biological tissues is primarily dependent on the lipophilic nature of the matrix [53–55].

6.4.1 Liquid–liquid (LLE) and solid phase extraction (SPE)

LLE and SPE have been used for many years [56,57]. A summary of the reports and techniques are given in Table 6.4. LLE is inexpensive to set up and simple to use. It covers a wide range of contaminants and concentrations and can be used both in the laboratory and in the field. The disadvantages are that it is labour intensive and as a manual technique it is more variable. It uses large volumes of solvent, requires large volumes of water at low concentrations, and is prone to contamination and the formation of emulsions, which are difficult to handle.

SPE was developed to allow the extraction to be undertaken in situ in the field which reduces the need to transport large volumes of water back to the laboratory. It is also applicable when isolating more sensitive contaminants, e.g. pesticides. In addition SPE is inexpensive, small and easy to use. Sampling can be automated and integrated into the overall extraction and clean-up system. Large volumes can be extracted with both the particulate and dissolved phases being sampled. However, care is required when removing any adsorbed materials or monomers from polymeric adsorbents, e.g. XAD resins. Blank values must be kept to a minimum, especially with samples from relatively clean areas. Breakthrough volumes must be established for each of the congeners and the filter can clog with particulate material in waters with high suspended solids.

6.4.2 Semi-permeable membrane devices (SPMDs)

Integrative, passive sampling of water and air for organic contaminants can be made using SPMDs [58]. SPMDs were first introduced by [59] as passive in situ samplers for dissolved organic contaminants.

Petty et al. [60] showed that the SPMDs could be used as highly effective passive air samplers for PCBs from laboratory air over a period of weeks. Ockenden et al. [61] demonstrated the potential for SPMDs as air samplers with good agreement between the calculated air concentrations from the analysis of the organics from the SPMDs and active samplers. Strandberg et al. [62] showed that SPMDs could be used successfully as a passive sampler to determine the organochlorine content of compost. Leonards et al. [63] used SPE for the trace-level enrichment of hydrophilic compounds such as CBs. SPMDs and C_{18} empore disks were used as extraction methods to mimic the accumulation of contaminants in organisms. These techniques have been used for the screening of organic micropollutants in municipal waste water. Water samples were taken from the streams and extracted with standard SPE methods. SPMDs and empore disks were placed for 2–8 weeks in the influent and effluent waste water streams. Simultaneously, empore devices were placed in 10 l bottles with influent and effluent water for 2 weeks. The contaminants were extracted from the SPMD and empore disks with organic solvents. All final measurements were made with gas chromatography with AED and mass spectrometry (MS), and liquid chromatography with MS.

The SPMDs are made of hydrophobic low-density polythene *layflat* tubing filled with a thin film of lipophilic materials such as triolein. The material is porous at the <10 Å level which allows smaller organic compounds to diffuse through the membrane while restricting

the passage to larger molecules. Part of the rationale behind the selection of triolein was that the lipid would mimic the neutral lipids present in fish gills and synthesise the uptake of small organic chemicals such as CBs. Workers have found that regardless of matrix type, (air [61], water, [64] or soil [65]) the equilibrium concentration was not reached in less than ca. 28 days for most compounds with a $K_{ow} > 4$ with the concentration increase following first order kinetics. This relatively long equilibrium time can pose problems where material might be damaged or changed in the environment over the sampling period. In particular the water sampling can be compromised by algal growth on the membranes which can change the transfer coefficients of the contaminants through the membrane.

6.4.3 Soxhlet

The Soxhlet has become the benchmark method against which most other extractions are compared for efficiency and efficacy. The advantages of the Soxhlet are that it is simple to use and to set up, relatively inexpensive, and parallel/batch extractions, e.g. 12–18 samples, can be undertaken with large sample masses, e.g. 100 g. The system is *automatic* in that once it is set up it can be left unattended with good reproducibility, once the cycle rate has been calibrated. However, the Soxhlet uses large volumes of solvent, and the preparation and subsequent solvent removal are wasteful and time consuming. It is difficult to integrate the Soxhlet into an automated method to include subsequent sample handling. It requires venting in a fume hood (health and safety) and the whole system is difficult to miniaturise.

The Soxhlet extraction has been extensively used for the extraction of non-polar and semi-polar compounds from a wide variety of environmental matrices [57]. The size of the system can vary, but the more common configurations use ca. 100 ml of solvent and 5–50 g of sample. After maceration and homogenisation, the sample is ground with sodium sulphate to bind the water present in the sample after leaving for 6–24 h. The dry powder is refluxed with the solvent for 4–18 h. It is essential to match the solvent polarity to the solute solubility. A combination of an apolar solvent such as pentane or hexane with a polar solvent such as acetone or dichloromethane (DCM) provides an efficient extraction of the CBs. Single apolar solvents do not extract all CBs from low fat, phospholipid, matrices [55], but may be sufficient for fatty matrices which contain primarily triglycerides. Saponification can be used prior to, or in conjunction with, solvent extraction. This is an exhaustive method, but due to the aggressive treatment, some more labile compounds such as some higher chlorinated congeners can loose chlorine atoms [22,55,66].

6.4.4 Soxtec or Soxtherm

The Soxtec or Soxtherm has been developed to overcome some of the disadvantages of the conventional Soxhlet. The Soxtec or Soxtherm are multi-port, e.g. six extraction systems, designed to semi automate the Soxhlet and to reduce the time and solvent volume used. There are three phases in the extraction using these devices. Initially the sample is immersed in the boiling solvent, typically acetone: hexane 1:1. The heat applied directly to the sample and solvent mixture increases the extraction efficiency and therefore reduces the time required by 4–10 h. The solvent volume is reduced below the level of the sample and refluxed to rinse the residual solvent from the matrix. Finally, the solvent volume is reduced to prepare the sample for the next stage in the analysis, which is normally a clean-up stage.

McMillin et al. [67] compared the Soxtherm with microwave assisted extraction (MAE) and sonication for the extraction of sand, clay and sediment samples.

6.4.5 Blender, ultrasonic and column percolation

The simplest extraction technique for solid matrices is to blend or ultrasonicate the sample with a mixture of polar and apolar solvents. Apart from the polarity of the solvent, the efficiency of the extraction is dependent upon the homogeneity of the matrix and the mixing–ultra sonication–blending–soaking time. A number of workers have use this method for both sediments, soils and biological tissues (Table 6.4). Blending/ultrasonication are also simple and easy to use and can be used with a wide range of samples. The disadvantages are similar to the Soxhlet in that these methods are difficult to automate, they are labour intensive and use large volumes of solvent.

A number of workers have successfully used column extraction [47,68], blending with an Ultra Turrax and ultrasonic extraction [8,69] as part of the method for CB analysis. These are well established methods with few recent innovations.

6.4.6 Supercritical fluid extraction (SFE)

A supercritical fluid has a low viscosity, high diffusion coefficient and, in the case of carbon dioxide, a low toxicity and low flammability, which are all qualities superior to the organic solvents normally used. The advantage of carbon dioxide is that it has a low critical temperature (31.3°C) and pressure (72.2 atm). Other less commonly used fluids are nitrous oxide, ammonia, methane, pentane, ethanol, dichlorofluoromethane and sulphur hexafluoride [57]. A great advantage of SFE is that the extracts are very clean and only require moderate additional clean-up. There are, however, limitations to this technique where lipids and CBs are not separated [70]. Initially samples with high fat contents were not extracted without the co-extraction of some lipids. The addition of different types of modifiers and the use of alumina, silica or silica with silver nitrate in the extractor have partially solved this problem for the most non-polar PHHs such as PCBs, PCDDs and PCDFs [71,72]. An evaluation of classic extraction techniques and SFE for the determination of PCBs has been reviewed [57].

SFE has been used to isolate CBs from a number of environmental matrices (Table 6.5). Recent developments have focussed on the improvement of the extraction efficiency and on the ability to trap and clean-up the SFE eluant in a single automated step. This combined system has been achieved primarily with the addition of methanol (MeOH) or ethanol (EtOH) as a solvent modifier to increase the dielectric constant of the solvent and by providing a solid in-line trap consisting of a C_8–C_{18} silica, silica or florisil. This configuration not only traps the eluant, but allows the co-extracted materials to be separated from the CBs. CO_2 alone is an insufficiently polar solvent to extract aromatic compounds from soils and sediments, due to the π–π bonding of the contaminants with matrices that have a high organic carbon content. The polarity of the extraction is generally increased sufficiently by the addition of ca. 2–3% MeOH or EtOH to the matrix or on-line in the solvent [73].

Ashraf-Khorassani and Taylor [74] made a comparison of modifier addition to the matrix in situ versus the modifier addition to the fluid for the SFE of CBs in river sediment (SRM 1939) (Table 6.5). They concluded that the SFE was between 20–30% more efficient than the US–EPA Standard Method 3550 which uses liquid solid extraction (LSE). Offline

TABLE 6.5

METHODS FOR THE SUPER CRITICAL FLUID EXTRACTION OF CBs IN SEDIMENT AND BIOTA

Application	Extractant	Conditions	Reference
River sediment (NIST 1939) and air particulates (NIST 1649)	CO_2 (100%)	40 min dynamic 0.7–0.9 ml/min, 50 or 200°C, trap acetone or DCM	[75]
Sediment and sewage sludge (BCR 392)	CO_2 (100%)	20 min static, 40 min dynamic 1 ml/min, C_{18} or florisil trap, 20 or 60°C	[77]
	CO_2 (100%), CO_2 (98%) + MeOH (2%), CO_2 (95%) + MeOH (5%)	20 min static, 30 min dynamic, 30 min dynamic, C_{18} or florisil trap, 20 or 60°C	
Sewage sludge (BCR 392)	CO_2 (100%), CO_2 (98%) + MeOH (2%), CO_2 (95%) + MeOH (5%), CO_2 (98%) + EtOH (2%)	dynamic 1 ml/min, C_{18} or florisil trap, 20, 65 or 78°C	[76]
Sediment (Rhône estuary)	CO_2 (100%), CO_2 (98%) + MeOH (2%), CO_2 (90%) + MeOH (10%)	1 ml/min, 15 min dynamic, trap 10 ml n-hexane	[82]
River sediment (NIST 1939)	CO_2 (85%) + DCM (15%)	60 min dynamic at 1 ml/min, 80°C, 200 kg/cm^2, trap 15 ml DCM	[80]
Industrial soil (BCR 481) and river sediment (NIST 1939)	H_2O subcritical (HPLC-grade)	1 ml/min, 250°C, 50 atm, 2–60 min	[81]
PCBs from river sediment SRM 1939	CO_2 modified with 200 μl methanol	10 min static, 30 min dynamic 1 ml/min, C_{18} trap 30°C	[74]

MeOH modification was more effective than in-line modification, because it used less modifier and the trap temperature was not critical with the off-line modifier, whereas control of the trap temperature was important, where in-line supercritical fluid modification was used. Langenfeld et al. [75] studied the effects of temperature and pressure on the SFE efficiency for CBs (and PAH) in river sediments. They found that the temperature was the most critical factor for the extraction of CBs. Above 200°C the extraction was quantitative regardless of the pressure range used ($\sim$150–650 bar). However, they only used pure CO_2 without a modifier. Much lower temperatures were required by other workers who subsequently added ca. 2% MeOH to increase the polarity of the supercritical fluid. Bowadt et al. [76] recommended florisil or ODS (C_{18}) to trap CBs with *n*-heptane as a trap eluant from the SFE of sewage sludge with CO_2 modified with <2% MeOH or EtOH. Extraction efficiencies were generally higher for the lower chlorinated congeners (CB28 and CB52). They recommended that the trap temperature was above that of the modifier. Similar methods were applied by the same group for the determination of CBs in harbour sediments and from the Venician lagoon containing high (1.5%) levels of sulphur [77]. The SFE technique avoided the need to apply alternative methods to remove the sulphur with tetrabutyl ammonium (TBA) sulphite [78] or copper. Gere et al. [79] have evaluated SFE as a bridge to automating sample preparation for GC, GC-MS and HPLC measurements of CBs in soils, sediments and leachates. They used CO_2 with a modifier during a 2 min static followed by a 30 min dynamic extraction at 4 ml/min, trapping the eluant on ODS Hypersil at 40°C. These workers also advocate using a lower purity of CO_2 than *ultra pure* since there is a reduction of ca. 50% per sample and a higher purity is only needed if the ECD is used rather than a MSD. Automated SFE can also aid the routine analysis.

Tong and Imagawa [80] used SFE to isolate the CBs in sediments. A SRM 1939 was extracted using CO_2 with 15% DCM at 1960 bar and 80°C with 60 min dynamic extraction. The values obtained by SFE compared favourably with those from the Soxhlet extraction. Yang et al. [81] demonstrated that supercritical water can be used to quantitatively extract CBs from soil and sediment. Normally the solubility of CBs in water is very low, ca. 0.5 μg/ l, at ambient temperatures and pressures. However, under supercritical conditions, e.g. 350°C and >2000 bar to dielectric constant is similar to DCM. At $\sim$100 bar at 300°C qualitative extraction was obtained in 5 min for both soil (CRM 481) and river sediment (NIST 1939). Fernandez et al. [82] developed an experimental design for the SFE of CBs (and PAHs) from sediment using a range of modifiers concentrations from pure CO_2 to CO_2 with 10% MeOH. A comparison was made with Soxhlet extraction. Whereas the SFE gave ca. 15% higher recovery than the Soxhlet method, the precision of the SFE was poorer (RSD 9% for SFE and 3.7% for Soxhlet, $n = 5$).

6.4.7 Microwave assisted extraction (MAE)

MAE uses an electromagnetic wavelength of 300–300 000 Hz. Most microwaves use a frequency of 2450 Hz. The electromagnetic field induces a rotation of molecules with a dipole moment without affecting the molecular structure [83]. MAS offers:

- fast (ca. 10 min with 12 vessels in parallel), efficient extraction with high sample throughput;
- reduced organic solvents including chlorinated solvents such as DCM and $CHCl_3$;
- improved safety for analysts;

• applicable in the laboratory, in the field or at sea.

Commercially available systems operated between 100 and 1000 Pa depending on the application. The extraction vessels are constructed of polyimide and/or fluorinated polymers which are transparent to microwaves. MAE has been reviewed [84] for a wide range of compounds, including CBs, and environmental matrices. MAE can be used in either closed or open containers in conjunction with solvents of high dielectric constants. In the closed container configuration the solvent, e.g. acetone–hexane (1:1), is heated above the boiling point of the solvent, typically at 115°C for ca. 10 min. Li et al. [85] evaluated rapid solid sample extraction using MAE in a closed vessel. They examined marine and harbour sediments, sand, soil and air filters. Between 1 and 5 g of sample were extracted with 30 ml acetone:hexane (1:1) at 115°C for 10 min. Recoveries were >80% with a RSD of ±10% at a concentration level of 0.5–2 mg/kg. Pastor et al. [86] used a mixture of water and toluene (10:1) on dried sediments (2 g) for 6 min at 660 W power in a PTFE hermetically sealed reactor. The results compared favourably with parallel Soxhlet extractions. This method uses yet smaller quantities of organic solvent and does not require either acetone or chlorinated solvents such as DCM.

6.4.8 Accelerated solvent extraction (ASE)[3]

ASE operates under elevated pressures and temperatures which increases solubility and allows the solvent volume to be reduced. ASE is relatively expensive, but shows more stable recoveries than MAE. No degradation of analytes was observed for either of the two techniques. ASE is a fast method of sample preparation compared with the conventional methods of Soxhlet blending/ultrasonic or column percolation. It requires small volumes of solvent relative to Soxhlet and the extraction is conducted at temperatures of 50–200°C and at pressures of 50–2000 Pa with extraction times of 5–15 min [87]. Following extraction the pressurised vessel is allowed to equilibrate for ca. 5 min after which the extract is flushed from the extraction cell. Ezzel [88] and Richter [89] reported this extraction method using elevated temperatures and pressures with DCM and acetone (1:1) to isolate PCBs from sewage sludge, from oyster tissue using iso-octane as a solvent and from the freshwater fish tissue using hexane. Heemken et al. [90] compared ASE with Soxhlet, sonication and methanolic saponification for the determination of organic contaminants in marine particulate matter. In this application acetone: hexane (1:1) was used as the solvent to compare the methods. Recoveries for the ASE were between 97 and 108% with a relative standard deviation of <8%. Schantz et al. [91] evaluated ASE of urban dust (SRM1649a), marine sediment (SRM1941a), New York/New Jersey waterway sediment (SRM1944) dried mussel tissue (SRM2974) and the Carp I and Carp II tissue aqueous slurry produced by NRCC. A comparison was made with Soxhlet with DCM, and with ASE using DCM, acetonitrile and hexane: acetone (1:1). In general there was little difference between either Soxhlet and ASE or between ASE with different solvents.

6.4.9 Limitations of SFE, ASE and MAE

Each of these techniques uses an extraction container which limits the sample size and

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[3] ASE is also reported as pressurised fluid extraction (PFE) Heemken et al. (1997).

therefore the ultimate detection limit of the method. The limited sample size can be a considerable drawback when analysing samples from relatively clean areas such as offshore sandy sediment samples or when extracting lean tissues with low concentrations of CBs. However, much of the CB contamination is associated with the organic carbon or soot particles in the fine fraction of the sediment. Therefore by wet sieving the sediment (or soil) through a <63-μm or <20-μm sieve [92] it is possible to concentrate the contaminants sufficiently so that they are amenable to extraction by one of the enclosed vessel extraction techniques. A critical evaluation of extraction techniques applied to persistent organic contaminants is given elsewhere in this book [52].

6.5 CLEAN-UP

Trace amounts of co-extracted materials such as lipids, wax esters and sulphur can degrade the analytical chromatographic column which is both expensive and time consuming to replace. These materials not only degrade the chromatographic resolution, but they can also co-elute to interfere with the measurement of the CBs. Effective clean-up of the sample extracts is therefore an essential part of the sample preparation prior to GC and LC-GC separation because even a trace of lipid (0.1 mg) will become significant if the final sample volume is reduced to ca. 100 μl. The clean-up for CBs falls into two categories: destructive and non-destructive. These techniques are well-developed and have changed very little in recent years with the exception of automation and the use of HPLC to replace some of the manual LC gravity clean-up columns [22,25,57]. Examples of these methods are summarised in Table 6.6.

Destructive methods are mainly alkaline treatment (saponification) or oxidative dehydration (sulphuric acid treatment). Alkaline treatment is similar to the saponification used in conjunction with extraction, but is applied sequentially to the solvent extraction instead of applying it to the matrix directly. Concentrated sulphuric acid is mixed with the lipid extract (usually in pentane or hexane) and after mixing for some hours the organic layers are separated from the acid in a separation funnel [93]. Alternatively sulphuric acid can be added to silica and the sample can be eluted from the silica chromatography column [47,66,94]. However, a fine carbon layer formed during elution tends to retain 2–3% of the PCBs present.

Non-destructive methods use solid-phase columns, gel permeation techniques and dialysis. Alumina columns are very effective and probably one of the most frequently used clean-up methods [55]. Columns of 10–20 g alumina have a fat capacity of ca. 250 mg. This capacity may be too small for the removal of the larger quantities of lipids associated with the mass of samples used for the analysis of non-*ortho* CBs. Silica and florisil columns are alternative adsorbents [95–97]. Gel permeation chromatography (GPC) or size exclusion chromatography (SEC) has also been used for lipid removal and the separation based on molecular size. SX-3 Bio Beads are used in most cases [47,98,99]. The disadvantage is that it is very difficult to obtain a 100% separation of lipids from the CBs. Often traces of the lipid which remain interfere with the measurement of the PHH resulting in the need for further treatment or a second GPC elution [100]. Dialysis techniques include the use of a polythene film of pore size ca. 50 μm. Around ca. 10 g of fat are dissolved in 15–20 ml cyclohexane and placed in the polyethylene tube. The CBs migrate from the fat through the polythene tube to

TABLE 6.6

METHODS FOR THE CLEAN-UP OF CBs

Application	Typical conditions	Reference
Destructive techniques		
Oxidative dehydration	Sulphuric acid:hexane or pentane. Shake	[93]
	Sulphuric acid–silica columns (50% loading). Elute with hexane	[66,94,112]
	Chromic acid	[152]
Saponification	20% MeOH:KOH 70°C for 30 min	[66,279]
	0.5 M EtOH:KOH 80°C 1 h	[152]
Non-destructive techniques		
GPC	500 × 25 mm i.d. Biobeads SX3 (200–400 mesh), ethyl acetate:cyclohexane (50:50)	[8,47,98,99,126,280–283]
Adsorption columns	Alumina, silica, florisil. Elute with hexane, pentane with or without addition of more polar solvent, e.g. ethyl ether, ethyl acetate	[50,95–97]
	Alumina:KOH	[284]
Combined columns	Alumina and silica	[50,285]
	30g silica/6 g phenyl ethyl RP/7 g silica. DMF–H₂O, petroleum ether, acetonitrile	[173]
HPLC	300 × 3.9 mm i.d. μBondapak aminopropyl silica. Elute with hexane	[286]
Dialysis	Polyethylene pore size 50 μm cyclopentane	[101]
Partitioning for lipid reduction	Hexane:acetonitrile	[287]
Sulphur removal	Mercury or mercury amalgam	[259]
	Copper	[288]
	Copper amalgam micro column	[289]
Jensen reagent	Fresh mixture of sodium sulphite and tetrabutyl ammonium (TBA) salts	[78]
	Sodium sulphite:alumina column	[290]
	Silver nitrate:silica	[259,291]

the cyclohexane surrounding the tube. Recoveries of more than 95% can be obtained [59]. A disadvantage may be the large volumes of solvent needed, because the solvent has to be renewed regularly.

Dialysis has been available for some time as a clean up method to remove lipid from extracts [101–104]. Strandberg et al. [105] used dialysis with a polyethylene SPMDs to remove up to 20 g of lipid in a single membrane in the preparation of biological tissue for the determination of CBs. They found that ca. 90–99% of the lipid can be removed in a single dialysis. Hess [41] used [^{14}C]CBs 122, 126 and 169 to study the recovery of CBs by dialysis. In a static system the recoveries could not be improved beyond ca. 80% over a 8–10 h period. However, by developing a dynamic dialysis system with the dialysis bag inside a Soxhlet the recovery was increased to over 95% in 8 h. The rate of dialysis was temperature dependent with the optimum temperature being around 43°C, above which the lipids were also dialysed at an unacceptable rate Fig. 6.2a. When a wide range of different CBs were tested [41] it was found that the efficiency of the transfer was dependent upon the molecular surface area of the CBs with the smaller CBs being more variable and more able to pass through the membrane (Fig. 6.2b). The widely differing dialysis rates dependent upon molecular size makes it difficult to optimise the conditions much beyond a first stage clean-up system.

Rimkus et al. [106] used GPC with SX-3 biobeads 200–400 mesh (340 × 25 mm i.d.) with ethyl acetate–cyclohexane (50:50) at 5 ml/min followed by an auto solvent change, concentration and injection onto a Hypersil silica 5 μm with a ternary solvent system of n-hexane–
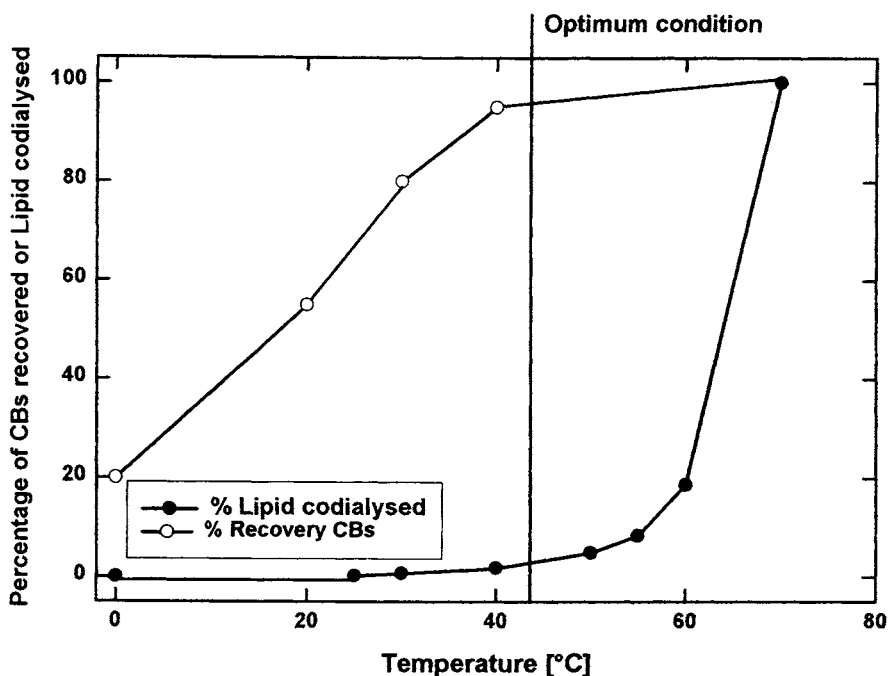
Fig. 6.2. (a) Temperature dependence of the dynamic dialysis of lipids and CBs using polyethylene 100-μm membrane pore. Hexane as solvent with a dialysis time of 8 h [41]. (b) Relative recovery of CBs by dialysis clean-up compared with the alumina/silica clean-up as a function of the molecular surface area [228] of the congeners [41].

toluene–acetone (88:10:2) to clean-up food and biological samples in a multi-residue scheme prior to GC-ECD or GC-MS analysis.

Sulphur must be removed from the sample extracts since the element is sufficiently soluble in organic solvents and, in large quantities, can completely saturate the detector signal, particularly the ECD. Sulphur is not only confined to soil and sediments. McKenzie et al. [107] found large quantities of [8]S extracted from the gut of green turtles which initially interfered with the analysis of CBs. Methods for the removal of sulphur from sample extracts are well established and given in Table 6.6. The separation of the CBs from sulphur can also be achieved using SFE [77].

6.6 GROUP SEPARATION

Group separation of the CBs is necessary (i) to concentrate the non-*ortho* CBs and the mono-*ortho* CBs that occur at relatively lower concentrations, e.g. CB105 and CB156 from the other congeners, (ii) to remove other interfering PHHs such as chlorobornanes (Toxaphene), polychlorinated naphthalenes (PCNs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzo-furans (PCDFs), polychlorinated diphenyl ethers (PCDEs) and their brominated homologues, and (iii) to remove further traces of co-extracted material remaining from the extraction of the bulk matrix. CBs should be separated from pesticides and other co-extracted micro-pollutants prior to GC separation since the chromatogram is generally sufficiently complex without additional interferences and

TABLE 6.7

HPLC SEPARATION OF NON *ortho*-CBs

Matrix	System	Conditions	Reference
Milk and animal fat	Shandon Hypercarb (7 μm) HPLC	Gradient elution acetonitrile–toluene 1 ml/min	[173]
Marine and freshwater fish	Shandon Hypercarb (7 μm) HPLC	97% Hexane–3% toluene, 50% hexane–50% dichloromethane	[50]
Fly ash	μLC 5 μm 2,4-DNPMP[a] in 0.32 mm × 400 mm	10% Heptane in pentane at 10 μl/min	[129]
Marine fish	Comosil 5μ PYE 250 mm × 4.6 mm i.d.	Hexane 1.5 ml/min at 9°C	[190]
Soils, sediments and incinerator ash	Comosil 5μ PYE 250 mm × 4.6 mm i.d.	Pentane 0.7 ml/min at room temperature	[131]
Aroclor and fish	Shandon Hypercarb (7 μm) HPLC	Hexane 2ml/min double forward and reverse flow	[132]
Marine mammals	Comosil 5μ PYE 250 mm × 4.6 mm i.d.	Hexane 0.5 ml/min at room temperature	[66]
Standards	Comosil 5μ PYE 250 mmμ4.6 mm i.d.	Hexane 0.5 ml/min at various temperatures	[128]
Sediments, mussels and plaice	Comosil 5μ PYE 150 or 250 mm × 4.6 mm i.d.	Hexane 0.5 ml/min, 15°C, forward and reverse flow	[41]

[a] DNPMP, dinitrophenylmercaptopropylsilica.

TABLE 6.8

CHROMATOGRAPHIC SEPARATION TECHNIQUES FOR DIFFERENT GROUPS OF CONGENERS

Congener	Group		
	Major mono-*ortho* CBs 105, 118 and 156	Minor mono-*ortho* CBs 114, 123, 157, 167 and 189	Non-*ortho* CBs 77, 126 and 169
Single techniques			
Single-column GC without pre-separation	Possible with care	Difficult	Currently not possible
LC (with diode-array)	Currently not possible	Currently not possible	Possible with care at relatively high levels
MDGC	Easy	Easy	Difficult
Hyphenated techniques			
LC-GC online	Difficult	Difficult	Difficult
Adsorption charcoal	Easy	Difficult	Difficult
PGC-HPLC-GC offline, PYE-HPLC-GC offline	Easy	Easy	Easy
LC^2-GC offline	Easy	Easy	Easy

possible misidentification. Silica gel columns of 1–2 g are frequently used to obtain such a pre-separation [55,96,97]. The CBs and chlorobenzenes are eluted with a non-polar solvent (hexane or iso-octane) in the first fraction. Other absorbents such as florisil have also been used [93]. The disadvantage of these gravity adsorption columns is that they are rather sensitive to moisture and the elution pattern must be calibrated regularly. Separation by HPLC on silica columns is more reproducible. Hess et al. [57] have focussed particularly on the separation and quantification of non-*ortho* CBs and the recent methods used to isolate these congeners are summarised in Table 6.7 and the advantages and disadvantages are given in Table 6.8.

Jansson et al. [8] developed a multi-residue scheme to separate CBs from PCNs, PCDDs, PCDFs and CHBs. The separation of CBs from organochlorine pesticides was made by increasing the polarity of the *n*-hexane eluant through a silica gel column with 5–15% of methyl *tert*-butyl ether [97]. The isolation of the non-*ortho* CBs is based primarily on the planarity of the molecule compared with the *ortho* CBs and as such these congeners tend to be separated along with other planar PHHs. The early developments used various forms of carbon or graphitic carbon to make these separations and the column materials used are referenced below.

Column material	Reference
Active carbon	[3,11,47,46].
Polyurethane foam impregnated with carbon	[108].
Glass fibre substrate-carbon	[109–112].
Active carbon HPLC	[99,113].
Silica gel/active carbon	[114,115].
Carbopack C mixed with Celite	[95,116,117].

Active coal on Chromosorb   [118].
Charcoal   [94,119–121].

    Activated adsorption columns were also investigated.
Florisil   [93,122–124].
Alumina/silica   [93,95].

Many of these developments have been extensively reported and reviewed previously
[22] and are summarised here and in Table 6.9 for completeness.

There are three techniques which are mainly used for the pre-fractionation of PCDDs,
PCDFs and planar PCBs. These methods use gravity carbon columns [11,95,108], HPLC
with graphitized carbon columns [50,125] and HPLC with PYE (2-(1-pyrenyl) ethyl
dimethyl silica) columns [41,126–128]. More recently, other HPLC columns have been
developed to isolate the planar contaminants from other PHHs. 2,4-Dinitrophenyl mercap-
topropyl silica (DNPMP) has been used as an electron-acceptor μLC stationary phase for
the separation on planar chlorinated hydrocarbons [129] as an alternative to gravity column
separation methods. Grimval and Östman [130] reported two additional HPLC phases for
the isolation of the mono-*ortho* CBs 77, 126 and 169. The three congeners were eluted on the
5-μm dinitroanaline propyl silica (DNAP) column as a single peak with *n*-hexane after 11.5
min at 1 ml/min and as an unresolved triplet after 8–10 min on the tetranitrofluorinimino
propyl silica (TENF). The three non-*ortho* congeners are separated from the other *ortho* CBs
and are eluted in a single small volume. These phases can be used as an alternative to the
pyrenyl silica (PYE) column in isolating the non-*ortho* CBs. When these congeners are
determined by GC, it is not necessary for them to be resolve from each other on the HPLC.

Huang et al. [131] evaluated the porous silica containing 2-(1-pyrenyl) ethyl dimethyl
groups used by previous workers [41,126–128] to provide another set of optimum separa-
tion conditions to isolate three *ortho* (CBs 77, 126 and 169), eight mono-*ortho* (CBs 105,
114, 123, 156, 157, 167 and 189) and two di-*ortho* (CBs 170 and 180) CBs using a Comosil
5-PYE 150 × 4.6 mm i.d. column with pentane at room temperature and flow rate of 0.7 ml/
min. The method was successfully applied to reference soils, incinerator ash and sediment
samples. Since most other workers have identified that separation on the Comosil PYE
column is temperature dependent, the term *room temperature* may not be sufficient without
recording the actual temperature of the system. Wells et al. [128] found that the temperature
of the column had a significant effect on the separation of CBs using the PYE column (Fig.
6.3). One of the more difficult separations between CB 138 and CB 163 on most GC
columns[4] was possible on the PYE column providing that the column temperature was
reduced to ca. 0°C. Qi and Anderson [132] used the porous graphitic carbon column to
separate CB 77 and CB 110 along with the other non-*ortho* and mono-*ortho* CBs using a
double forward and reverse flow at a constant flow with hexane as an eluant.

6.7 LC-GC COMBINATIONS

One of the main drawbacks in the determination of CBs in most matrices is the labour
intensity of the sample preparation and the data analysis. On-line LC-GC has been applied to
a number of determinations of CBs [133–135] in order to reduce the cost of the analysis.

TABLE 6.9

MULTI-COLUMN CHROMATOGRAPHIC SEPARATION OF CBs

Matrix/application	Column(s)	Configuration	Reference
CBs in air and soils	SFE–CO$_2$ - 50 m 0.32 mm SE-52	SFE-GC	[292]
Planar CBs in fish	10 m × 0.32 mm CP Sil 8 CB and 22 m × 0.22 mm HT-5	SGE column switching GC^2-MS with ^{13}C	[250]
Comparison of conventional and MDGC techniques	0.25 μm SE-54 and 0.25 μm OV 210	Siemens SiChromat-2 2MDGC-ECD	[152]
Separation optimisation	0.26 μm 5% CPSil-8 and 0.1 μm HT-5	Serial	[148]
Aroclors, fish, marine mammal tissue. Mono-*ortho* CBs	25 m 0.3 μm Ultra 2 and 25 m 0.3 μm FFAP	MDGC	[159]
Resolution of conventionally difficult separations e.g. CB28/31, CB138/163, CB149/118	30 m 0.15 μm Smectic Liquid crystal	Single	[150]
CBs, PCDDs and PCDFs in fly ash	5 μm DNPMP LC - 30 m DB-5	μLC-GC-MS	[129]
Fish oil and human adipose tissue	DB-5 or Ultra 2 50 m × 0.2 mm i.d. 0.1 μm and DB-17 or NB 1701 50 m × 0.2 mm i.d. 0.1 μm	GC-ECD parallel columns	[160]

With an *on-line* clean-up and separation it is necessary to remove all co-extracted materials by flushing with alternative solvents, as opposed to *off-line* methods where the adsorbent is disposable. For most applications *on-line* LC-GC or SEF-GC requires *heart-cutting*[5] the selected determinands while allowing the non-target materials to be switched to waste.

The disadvantages for this technique in routine system are that the *heart-cut* requires a separate calibration of the chromatogram for each sample type. This calibration is grossly affected by dirty, un-cleaned sample extracts and so this technique cannot ideally be used as a clean-up as well as group separation method. This technique is currently more suited to the measurement of the more abundant CBs, e.g. for monitoring purposes, and less amenable to the toxic congeners which occur at relatively lower concentrations. Where the sample extract is injected into the LC, in a *one-shot analysis,* it is critical that the whole system is optimised to obtain a complete measurement. This approach can increase the sensitivity of the method, which is useful where the mass of the sample is limited. However, it does limit replicate analyses.

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[4] The separation between CB138 and CB163 can now be achieved using the HP-5 stationary phase.

[5] *Heart cutting* describes the technique in MDGC of isolating a small fraction or fractions which elutes from the first column and is subsequently chromatographed on a second column. This may be applied to any hyphenated chromatographic technique, i.e. LC-LC, LC-GC or GC-GC.
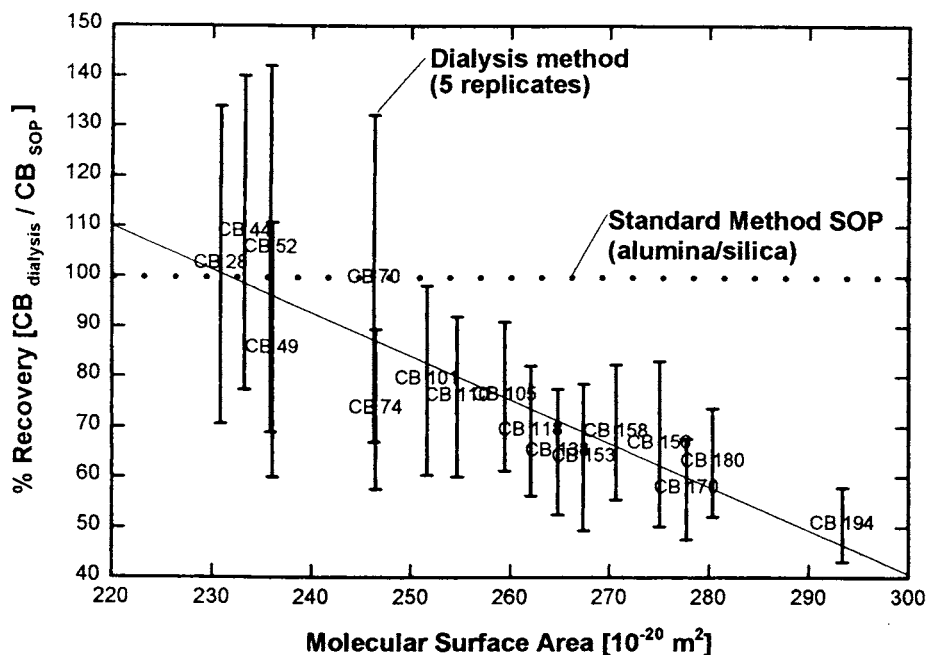
Fig. 6.3. The resolution between CB138 and CB163 on a pyrenyl-silica HPLC column, 250 × 0.46 mm i.d. as a function of the column temperature. The maximum resolution occurs around 0°C. The column cooling was controlled by a solid-state Peltier heat pump [128].

Linking LC with GC aids the development of the automated analysis and reduces the likelihood of contamination by enclosing the sample. There is also a substantial increase in the resolving power of 2D chromatography over the single system, especially where the polarity of the phases are *ortho*gonal. The preferred combination of columns can sometimes be difficult because the polarity of the phases and the solvents used can make the linking problematic. Interfacing the LC to the GC has also been hampered by the relative volumes of the carrier fluids (liquids and gases). Rather that have a continuous flow-through system, both difficulties in linking LC with GC can be overcome by using the modulated approach by cryofocusing the eluate from the first column, removing the first solvent before flushing onto the second column with an alternative fluid (i.e. a second solvent in the case of LC-LC or flash vaporizing into the carrier gas in the case of LC-GC).

Welch and Hoffman [129] developed a multi-dimensional μLC-GC-MS to analyse CBs, PCDDs and PCDFs in fly ash. The specificity of the μLC phase coupled to the high resolution MS ($R_s > 10\,000$) significantly reduced the likelihood of interferences from organochlorine pesticides, chlorophenols and their derivatives and PCDEs. Quantitative transfer with a precision of $\pm 17\%$ were obtained with a flow rate of ca. 10 μl/min from the μLC via an open-split interface.

In addition to providing a more cost effective analysis, automation may also be necessary where the sample size is limited. Most of the conventional sample preparative methods only use a fraction of the total sample in the final measurement, but in some applications the

whole sample is required. ubcutaneous adipose tissue taken by needle aspiration only provides small mg quantities of material, of which only 200–800 μl of sample are available for the determination of CBs. Gort et al. [136] coupled an on-line normal phase (hexane) LC-GC to provide automated clean-up, separation and measurement of CBs by ECD.

6.8 ANALYTICAL CHROMATOGRAPHY

6.8.1 Sample injection

Although sample injection is an essential technique for the effective transfer of the sample to the capillary column it is probably the least studied or optimised [137]. Grob pioneered the development and the optimisation of the injector for the GC yet he still comments that 'If an engine worked as unreliably as the capillary GC injector, our world would still be crowded with horse carriages'. There are three main injection techniques used in the analysis of CBs in environmental tissues, the splitless, the programmed temperature vaporizing (PTV), the splitless injector and the on-column injector. While the splitless injector is still used by most laboratories for CB analysis, it must be optimised for splitter time, needle length in relation to the length and volume of the injector, and the type and cleanliness of the injection liner [138].

The advantage of the on-column over the splitless injector is that the optimisation conditions are more straightforward and there is less opportunity for mass discrimination over the range of CBs compared with the splitless injector, primarily beacuse all of the sample injected is placed onto the column. However, this in itself can be quite problematic if the sample has not undergone a rigorous clean-up. McKenzie [42] identified a problem with the determination of CBs and OCPs in Minke whale blubber extract where wax esters proved difficult to remove completely. The uncleaned sample seriously affected the column performance when using an on-column injector, whereas analyses with a splitless injector were not affected since the wax esters were not sufficiently volatile to be transferred to the GC column. Only the glass liner required regular, routine replacement.

6.8.2 Chromatographic phases

Packed columns used in the 1970s [139] and early 1980s have been replaced initially by glass and then by higher resolution, polyamide coated, fused silica capillary columns [140,141]. Stationary phases of different polarities can be used for the determination of PCBs [142–145], but non-polar and medium-polar phases generally offer a higher resolution [146,147].

A number of stationary phases tailored to the separation of CBs (Table 6.10) have shown considerable improvement over the more conventional proprietary phases available. These phases have had greater success in separating additional congeners when used in series with more conventional columns. A 1,2-dicarba-*closo*-dodecacarborane polydimethylsiloxane (HT-5) column has been used in series with a CPSil-8 (5% diphenyl polydimethylsiloxane) column with helium as carrier gas to separate 84 congeners by ECD (108 by MS) [148]. The HT-5 column has an upper temperature limit in excess of 300°C enabling fast temperature programming and rapid (<60 min) analysis.

Larsen et al. [149] further optimised the application of the HT-5 phase by using a 60

TABLE 6.10

CO-ELUTION CBS ON CAPILLARY GC COLUMNS WITH DIFFERENT STATIONARY PHASES

Congener	HT-5	CPSil 5	CPSil 8	CPSil 19	CPSil 88	CPSil 8/HT-5
28	–	–	–	–	16	–
52	–	–	–	–	–	–
77	149	–	110	–	82/183/187	–
101	60	–	84	–	55	84
105	141	132	132	–	129	–
118	–	–	149	–	200/123	149
126	167/185/202	129	129/178	–	–	–
128	159/174	–	167	–	193/201	167
138	–	160/163	160/163	160/163/158	–	160/163
153	–	–	–	–	–	–
156	172	171	202/171	–	–	202
157	–	202	173/200	180/197	–	–
158	175/178	–	–	163/138	138/160	–
169	–	–	–	203/196	–	–
170	–	–	190	190	–	–
180	193	–	–	197	197	–
194	–	–	–	–	–	–

m × 0.25 mm i.d. fused silica column with a film thickness of 0.25 μm they were able to separate 106 congeners with ECD and 138 congeners with MS. This included the separation of the critical CB138/CB163. Berset and Holzer [150] tested a prototype smectic liquid-crystalline polysiloxane column for SUPELCO to separate some CB mixtures that have been more difficult to separate on more conventional columns such as CB28/31 and CB138/163. This phase was also useful to separate the non-*ortho* and mono-*ortho* CBs.

6.8.3 Multidimensional chromatography

Currently, all of the ca. 132 congeners detectable in formulations and environmental matrices cannot be separated on a single GC or HPLC column. However, the separation power can be substantially increased when columns are coupled either in series or in parallel. The greatest separation is obtained by using different systems and/or phase types to maximise the *ortho*gonality of the separation. Multiple columns such as serially-coupled columns [144] parallel-coupled columns [95,151] two-dimensional GC and, recently, comprehensive multidimensional GC (MDGC) have been developed to provide greater resolution of complex mixtures such as CBs [152]. Multi-dimensionality in GC has been reviewed by Geus et al. [153].

In two-dimensional GC co-eluting compounds are *heart-cut* as they elute from the first capillary column and are transferred to a second capillary column of a different polarity which is able to separate the isolated group of compounds [15,154]. This technique offers a complete separation of certain groups of unresolved congeners which are *heart-cut* from the first column. The technique is limited by the number of *heart-cuts* which can be made in one chromatographic run, since more than three to four lead to peak overlap on the second

column. Analysis times of the *heart-cut* two-dimensional GC are also relatively long. Nevertheless this technique has been applied to the determination of CBs in environmental samples [155].

The main drawbacks of multi-dimensional GC have been:

- a limitation on the number of *heart-cuts* possible in one GC injection;
- multiple, sequential injections are required to determine a large number of compounds of interest;
- time consuming;
- difficulty in obtaining quantitative data with high precision.

Comprehensive, modulation MDGC, [156] is a recent technique in which true three-dimensional chromatograms (2D time base × concentration) can be made [153,157]. Two capillary columns are connected in series by a retention gap which can be heated very quickly in a reproducible way. The eluants from the first column are cryofocused in the retention gap for a period of ca. 3 min. After which time the retention gap, which has a minimum thermal capacity, is heated quickly to *inject* the focussed compounds onto the second column of different polarity. The second chromatogram lasts for approximately the same period of time as the period of cryofocusing, so that a time series of 3-min chromatograms are produced to provide a two-dimensional separation time (Fig. 6.4). Because peaks collected at the modulator are very highly focussed before they enter the second column, an extremely high sensitivity can be obtained in combination with a very high selectivity. Details of the thermal modulator for comprehensive two-dimensional GC has been given by Phillips et al. [158].

Boer et al. [159] determined the mono-*ortho* CBs 60, 74, 114, 123, 157, 167 and 189 both in Aroclor mixtures, eel and seal tissue using MDGC with ECD combining the Ultra 2 and FFAP columns. This direct method was preferred to the separation of the mono-*ortho* CBs and the other congeners by HPLC. Either MDGC or off line HPLC of separation of the mono-*ortho* CBs is often necessary to reduce the risk of false positive results from interferences since these congeners occur at relatively lower concentrations compared with the di-and tri-*ortho* CBs. Hajslova et al. [160] used two capillary columns in parallel to separate 32 congeners, a further 19 organochlorine pesticides and 6 phthalate esters. The two columns were 5% phenyl methyl polysiloxane (DB-5 or Ultra 2) and either 50% phenyl methyl polysiloxane (DB-17 or 7% phenyl–7% cyano-propyl methyl polysiloxane (NB1701). Only fully resolved congeners were quantified on one of the selected columns for the fish and human adipose tissue sample analysed. The samples were cleaned-up using GPC with Biobeads SX-3 with chloroform as the mobile phase.

6.8.4 Chiral separation

The chiral separation of CB has recently been reviewed [161]. Of the 209 CBs, 78 display axial chirality due to the steric hindrance to free rotation about the C–C axis of the two aryl rings and of these 19 possess three or four chlorine atoms in the *ortho* position. The CBs 84, 88, 91, 95, 131, 132, 136, 149, 171, 174, 183 and 196 are present in commercial mixtures, four of which are present above the 2% level. GC separation of the 19 stable atropisomers have been made on the following columns:
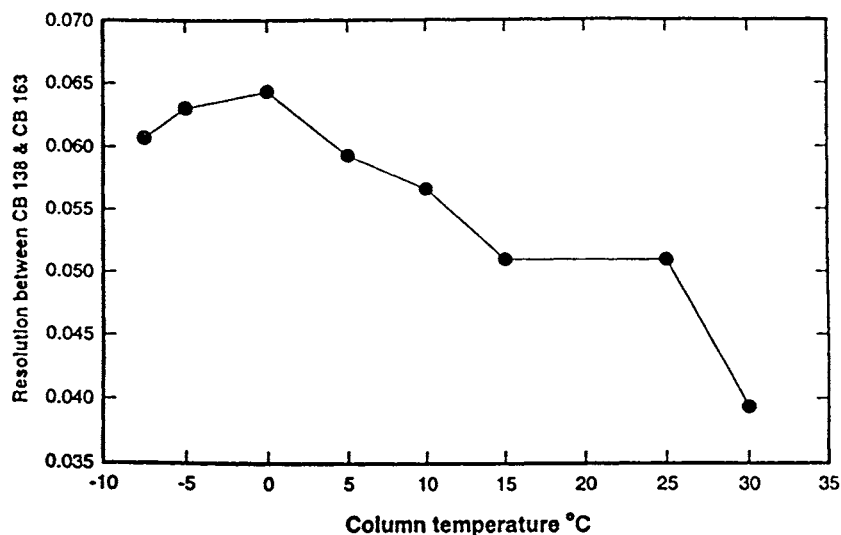
Fig. 6.4. GC-ECD chromatogram of CB77, CB126, CB169 showing the use of the thermal desorption modulator (TDM). The first column was 24 m × 0.2 mm i.d. 0.15 μm SB smectic phase, 24 cm 0.1 mm i.d. 0.12 μμm CP Sil 8 TDM and the second column a 5.3 × 0.2 mm i.d. 0.33 μm Ultra 2. The TDM was desorbed every 3 min. Reference [156] with permission.

- 2,3,6-tri-*O*-methyl-β-cyclodextrin [162];
- 2,3-di-*O*-methyl-6-*O*-thexyldimethylsilyl-β-cyclodextrin [163];
- 2,6-di-methyl-3-*O*-*n*-pentyl-γ-cyclodextrin [163];
- 6-*O-tert*-butyldimethylsilyl-2,3-di-*O*-methyl-β-cyclodextrin [164];
- *tert*-butyldimethylsilylated-β-cyclodextrin [165].

Haglund and Wiberg [166] have isolated enantiopure CBs by chiral HPLC and determined the retention order on Chirasil-Dex. The (−) CBs 84, 132, 136, and 176 eluted before the (+) enantiomers while the order was reversed for CB135 and 175. Glausch et al. [167] separated the enantiomers CB95, CB132 and CB149 in Clophen A60 using MDGC to *heart-cut* the congeners from the first column (DB-5) onto the second chiral phase, heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin (Chirasil-Dex). At lower concentrations none of these enantiomers were found in sediment samples taken from a river in southern Germany [168]. Blanch et al. [169] were able to determine the enantiomeric ratios of the chiral CB95, 132 and 149 in shark livers (*c. Coelolepis*) using MDGC. The achiral phases of the first column were DB-5 or OV-1701 with the chiral β-cyclodextrin, Chiralsil-Dex phase on the second column. Benicka et al. [170] separated the enantiomers CB84, 91 and 95 in technical formulations and in sediment and soil extracts using a dual column MDGC. The first column was a 25 m × 0.25 mm i.d., 0.12 μm CP Sil 8 and the second column 25 m × 0.25 mm i.d., 0.25 μm CP ChirasilDex CB with H_2 as carrier gas.

However, to date none of the CB enantiomers have been shown to be specifically toxic or able to induce any observable biological effect.

6.9 DETECTION

The power of the GC separation, especially with the thin (0.1 μm) film phases, must be matched by the sensitivity and the specificity of the detector to measure concentrations of CBs at the 1: 10^{-12}–10^{-14} level. These low levels of detection are required for the analysis of samples from relatively clean areas of the environment, for example the polar regions, oceanic and remote atmospheric samples. The are also required for samples obtained from small organs from single animals taken as part of biological effects studies or the investigative analysis of human adipose tissue.

A summary of the detectors which have been used for the determination of CBs is given in Table 6.11 along with the relative advantages, disadvantages, cost and current detection limits. Except for recent work, the details of those detectors that are well established, in particular the ECD and the mass spectrometer, are not given here. ECDs were used initially because of their high sensitivity for electronegative compounds, but mass spectrometric (MS) detection has become a routine method for measurement. Modern MS instruments are easy to operate, are more selective than the ECD, and have a better linear range.

6.9.1 Electron capture detection (ECD)

The most widely used detector for CB analysis is still the ECD. It is inexpensive, easy to use, highly sensitive and selective towards halogenated compounds containing one or more halogen atoms. Unfortunately the detector is not only sensitive to the number of halogen atoms, but also to the spatial configuration of the molecule and its cross sectional area. This means that the detector response is specific not only to each congener, but also to its enantiomers, if they exist. The detector also has a very small linear range, making it effectively non-linear requiring both constant and multi-level calibration, although the small (380 μl) frequency pulsed, constant current detectors have extended the linear range. Recently the micro-ECD with a cell size of ca. 150 μl has been developed to further improve sensitivity, linearity and dynamic range [171].

The normal make-up gases are either N_2 or CH_4/Ar with an optimum flow of ca 30–40 ml/min [172]. In general these make-up gases and the carrier gases are free from O_2. Even traces of O_2 will rapidly degrade the stationary phase on the GC column and affect the response of the detector signal. Lower flow rates for the make-up gas will increase the sensitivity of the ECD, but this also increases the non-linearity [173], which becomes more obvious by plotting the response/mass vs. mass [174].

In general, the ECD response can be greatly enhanced by the addition of small quantities of O_2. For example, 0.2–0.6% can increase the response of ethyl chloride by ca. >200× [175]. Noll et al. [176] studied the effects of an oxygen doped ECD on the analysis of CBs. Low chlorinated CBs produced negative peaks since compounds with weak electron affinities do not compete with dissociative ionisation by thermal electron detachment. The O_2 doping moderately increases the ECD sensitivity towards higher chlorinated CBs, but does not outweigh the increased detector noise. The temperature of the detector should be constant. The higher the temperature the less likely will be the contamination from compounds with low volatility eluting from the column. The ^{63}Ni detector is stable up to 320-340°C and can be cleaned in situ by replacing the make-up gas with H_2 and raising the temperature to ca 400°C for 30 min.

TABLE 6.11

DETECTORS USED FOR THE DETERMINATION OF CBs IN ENVIRONMENTAL MATRICES

Detector	Advantages	Disadvantages	Detection level	Relative cost	References
High resolution MS (HRMS)	Very high specificity and sensitivity. Provides spectral identification. Use ^{13}C-labelled analogues	Highly specialised operation. High capital and maintenance cost	Low, ng/kg	Very high****	[50,282,285]
Isotope dilution MS (IDMS)	Very high specificity and sensitivity. Provides spectral identification. Use ^{13}C-labelled analogues	Highly specialised operation. High capital and maintenance cost	5 pg/kg	Very high**	[95,115,273,293–296]
Low resolution (LRMS) quadrupole and MSD	Used primarily as an GC detector	Specialist operation. Moderate capital and maintenance cost	CB28 PCI 200 μg/kg. NCI 700 μg/kg.CB80 PCI 253 μg/kg. NCI 2.2 μg/kg	High****	[297]
Ion trap detector (ITD)	Used primarily as an GC detector	Specialist operation. Low and maintenance cost. Own ITD MS library	Low, μg/kg	Moderate****	[298–300]
Flame ionisation detector (FID)	Simple and easy to use. Good linearity. Useful as a check on clean-up efficiency	Poor sensitivity and specificity.	0.5 mg/kg	Low[+]	[301]

Atomic emission detector (AED)	Response only dependent on elemental composition. Robust and stable			Moderate***	[185–187]
Fourier transform infra red (FTIR)	Provides alternative structural information	Specialist operation. High capital cost	0.1 µ/gkg	High**	[302]
Electron capture detector (ECD)	Very sensitive. Inexpensive. Directly coupled to GC	Only selective for electron capturing material like halogenated hydrocarbons. Response dependent on molecular structure. Small linear range	0.1 µg/kg	Low****	[160,171]
Ultra violet (UV)	Directly coupled to LC. Fast, easy to use. Automated with clean-up	Lack of specificity and low sensitivity	1–10 mg/kg	Low**	[190]
Diode array detector (DAD)	Direct coupling to LC. Fast, easy to use. Automated with clean-up. Good screening detector	Lower sensitivity	10–100 µg/kg	Moderate**	[190]
ELISA	Fast, effective screening tool for contaminated sites	Some false negatives. Relatively insensitive	20–100 mg/kg	Low***	[195,196]

Scale: **** very applicable to ** not very applicable, + generally unsuitable.

6.9.2 Mass spectrometry

The technique is reviewed on regularly by Burlingame et al., [177,178]. The mass spectrometric detector (MSD) is fast becoming the preferred alternative to the ECD. The simpler MSD and ion trap detectors (ITDs) have been fully integrated with the capillary GC and most instruments now have the fused silica column terminating inside the ion source of the MS. The ITD differs from other MS techniques in that the ions formed from the ionisation trapped electronically and then destabilised according to their mass and transferred to the electron multiplier outside the trap itself. Full scan spectra are possible at low concentrations (low μg/kg) and the detection limit can be decreased further by ca. 2× when scanning over a narrow mass range. This approach is akin to single ion monitoring (SIM) using the MSD. The MSD is now stable and robust and although it requires more specialist attention than the ECD, it does offer considerably more power in terms of sensitivity, selectivity and confirmatory analysis. The MSD has a much wider linear range than the conventional ECD used for much of the routine CB analysis.

The high resolution MS provides highly specific mass detection with resolution between 6000 and 10 000 provided by accurate mass marking with PFK at m/e 316.9824 to prevent mass drift. This not only offers a high specificity, but also considerably reduces the likely interference with other trace contaminants such as PCDDs and PCDFs. While this overcomes most of the problems with false positives, errors associated with false negative interference may still exist. Samples that are not sufficiently cleaned-up and contain quantities of co-extracted materials which elute from the chromatographic column will competitively reduce the ionisation of the determinand in the ion source and so decrease the signal of the CB. This can be detected by observing the stability of the lock mass if it is used.

A further advantage of MS is provided by the increased sensitivity of the negative ion chemical ionisation (NICI) with the molecules containing more that four chlorine atoms. The sensitivity can be further enhanced by operating in the SIM or multiple ion mode (MIM) as opposed to the total (full scan) ion mode (TIC). The main disadvantage with using the MS in SIM or MIM is that the confirmatory power of the technique is considerably reduced. NICI has been used in a number of studies for the determination of CBs in the environment [179–181] In this mode the CBs generate a strong molecular base peak with a limit of detection (LOD) of 40–100 fg. Leonards et al. [182] combined the GC with an ITD in the MS/MS mode to detect mono-*ortho* CBs in biota and sediment at 60, 300 and 200 fg for CB77, CB126 and CB169, respectively. One of the main advantages of MS, in addition to sensitivity and specificity, is the ability to use ^{13}C-labelled CBs as internal standards to compensate for losses during sample preparation, especially at the fg level. Using [^{13}C]congeners reduces the need for extensive recovery experiments or having to apply recovery corrections in the method validation and improves the overall variance of the data [41]. There are several MS techniques which can be used for the determination of PCDDs, PCDFs and planar PCBs [183,184]. High resolution instruments, using electron ionisation, are normally required for the determination of PCDDs and PCDFs. Low resolution instruments, using negative chemical ionisation, may be used for the determination of planar PCBs.

6.9.3 Other detectors

The AED is a well established and widely used detector for elemental analysis. Although the AED has a excellent sensitivity for most elements, it has a low relative sensitivity for halogens. As a result it has tended to be overlooked as a detector for trace organic contaminants in favour of the ECD and more recently the MSD. The ECD has been used widely, primarily because of its simplicity of operation, its high sensitivity towards halogens and because of its low cost. However, the main disadvantage of the ECD is that it has a strong structure related response which requires each individual compound for specific calibration. The relative instability of the ECD also requires careful optimisation and frequent calibration each day or with each batch of samples. In contrast the AED is relatively very stable and has a common molar response for compounds of equal halogen content.

Pedersen-Bjergaard and Greibrokk [185] have reviewed the application of the capillary GC coupled to the AED to the analysis of environmental samples and [186,187] specifically studied the use of the AED for the analysis of CBs. The commercial AED instrumentation, with a detection limit of ca. 250–400 pg, was too insensitive for the determination of CBs in all but the most contaminated samples. The AED development was based on a 350 kHz on-column RF plasma set at 837.6 nm for the Cl emission operating at 350°C with He at 10 ml/min as the make-up gas and O_2 as the plasma dopant. The capillary column was positioned so as to sustain the plasma *inside* the end of the column. This *on-column* configuration improved the detection limit by 30 × and provided sufficient sensitivity to be compared directly with the ECD in the analysis of real sediment samples.

The AED is:

- very stable and not easily contaminated;
- easy to calibrate and maintain calibration;
- modified AED sufficient sensitivity to compare with GC-Fourier Transform infra-red (FTIR) and full scan MSD [186,188,189];
- applicable to wide range of organic and inorganic compounds.

6.9.4 Diode array detector

Krahn et al. [190] screened for non-*ortho* CBs in fish tissue. The analytes were extracted with hexane: pentane (1:1) and the CBs first separated on adsorption columns and eluted with DCM and hexane (1:1) and sequentially chromatographed using a Comosil PYE 5-μm column cooled to 9°C and measured with UV and DAD. The LOD for the DAD was ca. ten times less sensitive that the GC or GC-MS. However, the advantages of this technique over conventional UV are that the DAD allows the identification of individual analytes by comparing the spectra and establishing spectral homogeneity and, although less sensitive, the HPLC-DAD is rapid, cost effective and ideal for screening purposes.

6.9.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA has been applied to the determination of PCBs in a kit for screening purposes [191] and has also been evaluated by a number of workers [192,193,194] to evaluate the applicability of ELISA to the determination of PCBs and a comparison with other conventional methods. The technique uses an anti-PCB antibody, stabilized, preserved and immo-

bilized on paramagnetic particles, a PCB enzyme conjugate, e.g horseradish peroxidase-labelled PCB analogue plus the sample extract. This cocktail is incubated for ca. 30 min after which a chromogen is added (e.g. 3,3′,5,5′-tetramethyl benzidine) to provide a colour for spectroscopic measurement.

The advantages of the ELISA technique for the measurement of PCBs are:

- fast and cost effective compared with conventional methods;
- applicable in the laboratory and the field;
- available in kits for screening;
- single total value for legislative purposes.

The main disadvantages are:

- less sensitive that conventional GC-ECD/MS methods;
- narrow detection ranges so that the diluted sample extract must be re-analysed;
- false negative peaks are possible resulting from interferences;
- not congener specific.

Lopez-Avila et al. [195] compared the determination of PCBs in soils and sediments by MAE followed by GC-ECD and ELISA and found good agreement between the two techniques for reference materials as well as the soil sample obtained from the Superfund site in the concentration range 20–579 000 mg/kg. The ELISA screening method had a throughput of ten samples per hour giving considerable saving in both time and cost.

Johnson and Emon [196] evaluated the application of the ELISA method to determine the total PCBs in environmental sediment and soil samples. A comparison was made between ELISA and conventional Soxhlet, SFE with MeOH as a solvent, for contaminated samples with concentrations of CBs above the 5 mg/kg level. ELISA compares closely with conventional methods using clean-up and GC-ECD for the final separation and measurement. However, the *quick shake* method with MeOH, as recommended in many of the PCB diagnostic kits, followed by ELISA was found to be suspect giving low values as a result of incomplete extraction rather that biased results due to the ELISA technique. ELISA was shown to be a valuable, quick screening method with an LOD of ca. 5mg/kg.

6.10 QUALITY ASSURANCE

Once a procedure has been developed and the performance criteria established for the CB analysis, then it is necessary to maintain the validation by routine quality control procedures [197,198] and an appropriate external quality assurance scheme.

Most of the variability of the CB measurement still comes from the accuracy of the calibration of the GC and the calibration solutions. Instrumental variability can be reduced with internal standard and retention index markers [97,199]. The 2,4-dichlorobenzyl alkyl ethers [97] and the 2,4,6-trichlorophenyl alkyl ethers [199] are detectable by flame ionisation detector (FID), ECD and AED as well as having a suitable intense base m/e for SIM. Once the GC retention index of each congener has been determined and identification confirmed by reference to the mass spectral library (RSC, 1991, MS for Windows v 1.1A NIST, 1995) then the compound can be quantified against a standard solution of known concentration. External standards used to calibrate the GC detector for individual congeners

are prepared as calibration solutions from crystalline solids or liquids of high purity (>99%) which are available as Certified Reference Material (CRMs) (National Research Council of Canada NRCC (http://www.nrc.ca), Food and Drug Administration FDA (http://www.fda.-gov/ora/science/refs/lpm), Environmental Protection Agency EPA, (http://www.epa.gov) or as Standard Reference Materials (SRMs) from the National Institute for Standards and Technology (NIST) (http://ts.nist.gov/srm).

Errors associated with calibration can be greatly reduced by implementing the following guidelines [22,174,200].

- Only use certified solids or solutions. These are available for most congeners.
- Confirm the identity of the material(s) provided by MS.
- Control the preparation dilution and storage of calibrants by weight.
- Ampoule stock and working calibration solutions. Store in a cool dark place. Avoid using screw top containers and do not store screw top containers in the refrigerator.
- Confirm the purity of all solvents used by concentrating to ca. 100 × and analysing on ECD and FID.
- Calibrate the detector with sufficient frequency to ensure the response factor for each congener is ca. $< \pm 5\%$ of the actual response.
- Check the frequency of calibration, which is a function of the cleanliness of the GC system.
- Check new stock solutions against previous calibration solutions or against independent solutions of known quality.

Since many capillary GC analyses can take up to 1–2 h per sample to complete, it is essential to minimise the time taken to calibrate the GC and run the system checks (e.g. blanks). A summary of calibration methods is given in Table 6.12. Most GCs have integrated data systems and software to control and provide all data acquisition, integration and data output as well as controlling the GC programme conditions. Under such conditions it is easy to overlook essential data evaluation at the acquisition stages. Evaluation of the integrity of the data at a later stage in the database for example, is too far removed from the potential source of the error. A full manual check and calculation on the data should be made periodically. The manual check is a current requirement for accreditation by the United Kingdom Accreditation Service (UKAS) to confirm the GC instrumental operation. Many of the gross errors (e.g. ×1000, 100, 5 and 2) which occur in the results from External Quality Assurance Schemes such as the Quality Assurance of Information in Marine Environmental Monitoring in Europe (QUASIMEME) [201] occur as result of incorrect reporting units or calculation errors.

The range of the GC instruments capability is primarily dictated by the capacity of the stationary phase and the detector. Highly efficient thin film columns (ca. <0.1 μm) have been developed for maximum resolution and can set the upper limit of the GC working range with the lower limit set by the LOD, defined as 3σ of the baseline signal [202] and the limit of the determination of quantification is set at 10σ. A *reporting limit* set at a concentration below which the uncertainty of the measurement becomes unacceptable for the purposes of the analysis should be used.

During the last 10 years specific attention has been given to the quality of the analysis. There have been several worldwide inter-laboratory studies conducted for PCBs. Boer et al. [203] reported on a stepwise series of interlaboratory studies for ca. 50 laboratories world-

TABLE 6.12

SUMMARY OF METHODS OF CALIBRATION

Type	Use/advantage	Misuse/disadvantage
Single point calibration	Semi-quantitative, screening technique, calibrant and sample within ca. ±5–10% linear detectors	Inaccurate, especially at low concentrations. Not suited to the ECD
Bracketing standards	Small linear range of the ECD. MS detection	Extrapolation beyond the upper and lower limits
Multipoint calibration	Must accurate method for ECD. Requires quadratic or cubic spline type curve fit	Time consuming in use, maintenance of calibration solutions
Labelled internal standards ^{13}C, ^{2}H	Mass spectrometric detector, compensates for all recovery losses	Not suitable for ECD. Does not give any information on the intrinsic efficiency of the method

wide to determine individual CBs in sediment, fish and marine mammal tissue. The studies found an agreement of ca. ±50% for sediment and marine mammals tissue for CB138, 153 and 180. There were no significant differences in the results for the analysis of cleaned and uncleaned extracts. Results were generally poorer for lean muscle tissue. The *between-laboratory* standard deviations obtained by the more experienced laboratories which analyse these compounds are in the range of 10–25% [184,203–205]. A few certified reference materials (CRMs) have become available during the last years, but more are required [206].

Fuoco et al. [207] have reported their internal QA/QC procedures for the determination of CBs using reference materials from the European Union Standards, Measurement and Testing Programme, NIST and NRCC and the QUASIMEME laboratory Performance Studies, They concluded that there were a number of improvements made as a result of participation in an external improvement/laboratory performance scheme as well as having an internal QA system. Misidentification of CBs from data obtained by a single GC phase is possible. Gankin et al. [208] have developed the use of dual column analysis, with dissimilar phase polarity, and a quantitative structure retention model to identify the CBs measured by GC-ECD. This approach builds on the existing EPA method 8081 for compound identification and is an alternative to the MDGC or modulated MDGC for full chromatographic separation of the congeners. Galceran et al. [209] implemented a series of training programmes for the improvement of measurement of CBs in environmental and food samples amongst a group of Spanish laboratories. These programmes provide essential information in national and international QA programmes.

- An objective assessment of the quality of the data and the level of between laboratory agreement.
- A stepwise improvement programme as an interlaboratory training aid.
- The development of networks of laboratories undertaking CB analysis.

6.11 DATA INTERPRETATION

Interpretation of data is an essential part of chemical analysis. However, the extensive analysis of environmental samples for CBs over the years has produced a vast quantity of data, much of which is of doubtful or unknown quality. More recently, the availability of good calibrants, CRMs and external QA schemes have improved the reliability of data for many laboratories. The determination of CBs with appropriate QC/QA has resulted in extensive valuable data sets. These data have allowed a more detailed evaluation of the environmental impact or significance of these contaminants [210].

An additivity model has been developed to provide a link between possible cause and biological impact at low concentrations for a number of the toxic PHH compounds including planar CBs, by relating their ability to bind to the Ah receptor with that of the 2,3,7,8-TCDD. The details of these developments have been reviewed elsewhere, along with the resultant TEFs, for seven PCDDs, ten PCDFs [211,212] and for three non-*ortho* CBs, eight mono-*ortho* CBs and two di-*ortho* CBs [40]. The TEFs for each toxic congener are multiplied by the concentration of that CB to provide a toxic equivalent concentration (TEQ). The summation of these compounds, in terms of their toxic equivalent concentrations (TEC) to 2,3,7,8-TCDD provides a total toxic equivalence (TEQ) which may be used as an indication of their contribution to the Ah binding and subsequent EROD induction. A review of TEFs for humans and wildlife has been made by Berg et al. [213].

These TEQs have also simplified the link between the analytical scientist, the regulatory authorities and the policy makers to provide a single value for the equivalent CB impact, in association with other chlorinated compounds, such as dioxins and furans. These TECs are the *best available* values to express the toxicological impact of CBs, but may not reflect the true toxic impact. Although they often account for a significant proportion of the PHH input to the TEC [41] they do not account for the total TEC in an environmental area, much of which may be as a result of other anthropogenic sources, such as PAH and naturally occurring compounds [214].

Much data has been collected for many congeners to provide information on the patterns and the distribution (*chemical fingerprints*) of CBs, particularly in sediments, soils and biota [48,215–221] which reflect the inputs to the environment modified by the physicochemical properties of each CB and their propensity to be metabolised and modified. Such sets of data are rarely normally distributed and are not readily amenable to standards descriptive statistics. A simple overview is often obtained by expressing the data with median, range and the distribution by using the box and whisker plots [222]. A schematic diagram of data analysis as applied to CB information is given in Fig. 6.5. Before these data are analysed any further they are usually normalised in one or more of three possible ways:

- log transformation, which allows the data to be analysed by parametric as well as non-parametric statistics. Statistical calculations based on log transformed data substantially improves the homogeneity of the variance [210];
- normalisation to a co-factor such as the lipid content of the biological tissue or the total organic carbon content of the soil or sediments. A variety of co-factors have been use both to reduce the heterogeneity of the distribution either within different tissues of a species or within an environmental area, in order to establish absolute rather than relative trends in contaminant levels;
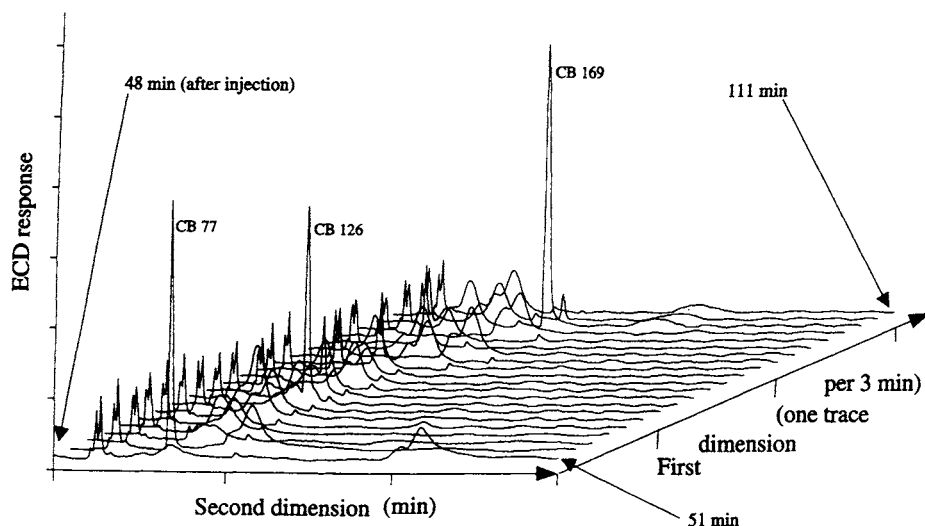
Fig. 6.5. A schematic flow diagram of the data analysis using normalisation and multivariate techniques.

- normalisation to a single reference congener such as CB153 to study the underlying contaminant patterns [66,210,223].

Variations in the CB concentrations as well as the pattern can be readily evaluated using principal component analysis (PCA). PCA is a valuable data reduction technique when there is a requirement to study the underlying trends in a large, multivariate data set. The data are *ortho*gonally transformed to provide the principle component (PC) which is a linear combination of the original variables, i.e. CB concentrations to explain the variance in the data. The PCs data cluster when samples have similar patterns [224]. Normally most of the variance is explained by the first two PCs which can be plotted to provide a graphical representation of the data. These biplots [225] have the following features:

- data in close proximity have similar properties, i.e. concentrations or relative concentrations;
- the circle represents the total variance;
- the length of the vector represents the percentage of the variance explained by the PCS which are plotted;
- determinands in the same area of the plot are correlated;
- determinands opposite each other are negatively correlated.

An example of the application of PCA to the evaluation of CBs in marine biota is given in Fig. 6.6. Plaice and mussels from the same area in the inner Firth of Clyde, west Scotland, have distinctly different patterns of CBs which would be difficult to distinguish from either the data tables or by comparison of the chromatograms. In Fig. 6.6 the upper PCA biplots of the first two PCS account for most of the variance of the data (PC1 73% and PC2 15%) of the CBs, with the exception of CB156. The clear differences between the plaice and the mussels

from the same area are due to the abundance of the more chlorinated hepta and octa congeners, CB170, CB180, CB187 and CB194 in the plaice. These difference are not due to inputs to the area, but to the different mechanism of uptake by the two species as a function of the log K_{ow} of the CBs. The more highly chlorinated congeners are less water soluble and therefore not taken up by the filter feeding mussels to the same extent as via the food of the plaice.

In the lower biplot in Fig. 6.6 there is a clear distinction between the CB patterns in the mussels from the inner and outer Firth of Clyde. The mussels from the outer Firth were associated with higher levels of the lower chlorinated congeners which, being more water
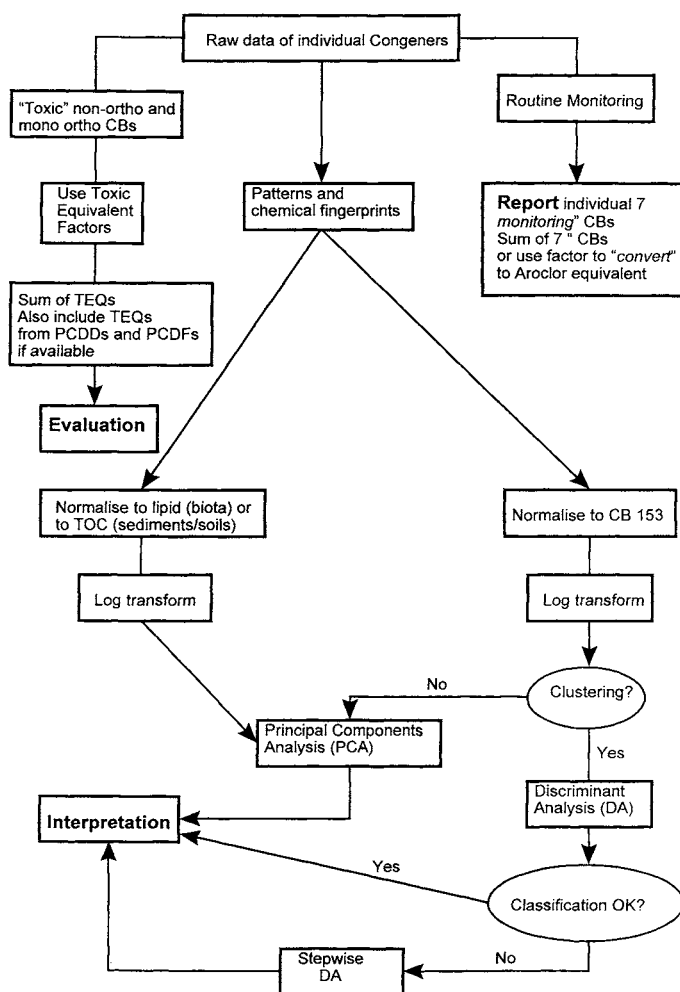
Fig. 6.6. Upper PCA biplot: chlorobiphenyls in mussels (M6 and M7) and plaice (F3) in the inner Firth of Clyde, west Scotland. Lower PCA biplot: chlorobiphenyls in mussels from the outer Firth of Clyde (M1 and M2) and the inner Firth of Clyde (M6 and M7).

soluble are more mobile and have a higher relative concentration at sites remote from the source. Similar patterns were found in the sediment from the same areas.

PCA, discriminant analysis and stepwise discriminant analysis have been applied to the data on the levels and patterns of CBs in marine mammals stranded in northern European waters. By using these multivariate methods it was possible to distinguish between the patterns of CBs in 12 different species of cetaceans and pinnipeds. The main differences in the patterns of CBs was due to differential metabolism and feeding behaviour [42,107,226, 227].

Evaluation of data by these multivariate techniques provides an elegant method of transforming extensive quantities of chemical measurements into key information to extend the knowledge and understanding of the impact of these complex mixtures on the environment.

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ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
Ab	antibody
Ag	antigen
Ag*	labeled antigen
anti-IgG	antibody generated against immunoglobulins
AP	alkaline phosphatase
B	absorbance
B_0	zero control absorbance
B_{excess}	standard excess absorbance
BSA	bovine serum albumin
CA	coating antigen
cDNA	complementary deoxyribonucleic acid
CDR	complementary determining region
CLIA	chemiluminescence immunoassay
CONA	conalbumin
CR	cross-reactivity
CV	coefficient of variation
DDT	1,1,1-(trichloro)-2,2-bis(p-chlorophenyl) ethane
DMSO	dimethylsulfoxide
EIA	enzyme immunoassay
ELIFA	enzyme-linked immunofiltration assay
ELISA	enzyme-linked-immunosorbent assay
EMIT	enzyme multiplied immunoassay techniques
EPA	Environmental Protection Agency
ESI–MS	electrospray ionization mass spectrometry
ET	enzymatic tracer
Fab	antibody binding site
Fc	crystallized fraction
FIA	fluoro immunoassay
FIIA	flow injection immunoassay
FILIA	flow injection liposome immunoassay
FT-IR	Fourier transform infrared spectrometry
GC–FID	gas chromatography–flame ionization detection
GC–MS	gas chromatography–mass spectrometry
GO	glucose oxidase
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
IA	immunoassay
IC_{50}	concentration inhibiting 50% of the absorbance produced at zero-dose
Ig	immunoglobulin

KLH	keyhole limpet hemocyanin
LC–DAD	liquid chromatography–diode array detection
LC–PCN–FD	liquid chromatography–postcolumn reaction–fluorescence detection
LDD	least detectable dose
LIA	liposome immunoaggragation assay
LIC	liposome immunoreaction assay
MAb	monoclonal antibody
MALDI–TOF–MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
mRNA	messenger ribonucleic acid
OVA	ovalbumin
PAb	polyclonal antibody
PAH	polyaromatic hydrocarbon
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PCR	polychain reaction
RAb	recombinant DNA antibody
RIA	radioimmunoassay
SFE	supercritical fluid extraction
SPE–LC–DAD	solid phase extraction–liquid chromatography–diode array detection
TG	tyroglobulin
TNBS	trinitrobenzenesulfonic acid
βG	β-galactosidase

7.1 INTRODUCTION

Environmental contamination is recognized as a worldwide problem. Part of this problem is caused by the application of hundreds of different compounds that are being used as pesticides in agriculture, horticulture and forestry. Inherently, they show a certain degree of toxicity and especially the less degradable, more persistent compounds present a problem. Residues of pesticides have been found in all kinds of environmental samples. Much research has been done to develop new and improve existing methods for pesticide analysis. On the other hand, a large number of industrial compounds such as aromatic compounds, polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and phenolic compounds are highly toxic and widespread environmental pollutants.

The methods generally used to measure pesticides and industrial pollutants are based on chromatographic techniques involving extraction, extensive purification and often application of derivatization procedures. Therefore, experienced personnel and expensive equipment are required in order to carry out this work. Immunoassays (IAs) are analytical tests that utilize antibodies (Abs) as specific recognizing elements. Their use for both qualitative and quantitative analysis has proved to be one of the most productive technological contributions to medicine and fundamental life science research in the twentieth century.

IA technology is not new, since the principles of IAs were first expounded by Rosalyn Yalow and Solomon Berson in 1959 [1] determining insulin in blood. Since then, there has been an exponential growth not only in the range of applications to

which it has successfully been applied, but also in the number of novel and ingenious assay designs. Such technology has not been confined to medical diagnosis, finding applications also in the forensic, veterinary, pharmaceutical, food and environmental sciences.

Ercegovich [2,3] first proposed the use of immunochemical techniques for environmental analysis in the 1970s, mainly focusing on radioimmunoassay (RIA). Some years later, Hammock pointed out on the potential and advantages of using enzyme immunoassays (EIAs) for the environmental monitoring of pollutants. Over the following years a great number of articles have appeared describing the development of EIAs for detecting trace amounts of chemicals in the environment [4–12]. However, the acceptation of IAs for environmental applications by analytical chemists has been rather slow, although some authors have noted the potential of these technologies [13–15]. It has been only during the 1990s that the scientific community and regulatory agencies have started to evaluate and recognize the advantages of IAs [16,17]. Nowadays, an important number of IAs for pesticides are commercially available (for the features of some of these assays see Tables 7.2 and 7.3, respectively). The US Environmental Protection Agency (EPA) has validated some of these assays and included 13 of them in the SW-846 methods list (see Table 7.4).

IAs exploits the ability of Abs to selectively and reversibly bind organic molecules. The other key reagent in most environmental IAs is the labeled ligand or competitor. IAs owe their versatility to the immune system ability to produce Abs in response to virtually any foreign molecule. For small analytes, such as pesticides, the compound of interest must be conjugated to a large carrier molecule, such as bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), etc., to render it immunogenic.

However, even if the method appears to be very easy and simple, an understanding of its basic principles is required to make a good use of an IA. Special attention has to be given to the cross-reactivity (CR) phenomena, matrix effects and to the data interpretation and validation procedures.

In summary, immunochemical methods are powerful analytical techniques that can answer many questions concerning environmental contamination. Both the availability of commercial IA test kits and the fact some IAs have already been included in the list of SW-846 methods of the EPA indicate that this technology is finding its place in the environmental area as an effective screening method to complement other analytical techniques. Although it is a relatively new technique for pesticide residue field-testing and for the screening of industrial pollutants, IAs appear to hold great promise. Additional advantages are the reduction of the costs caused by shipping negative samples and the possibilities to provide test results without delays. Most of the IA tests currently described are designed to be sensitive enough to assay water samples directly without concentration or clean-up steps. Future developments on immunosensor systems are a great promise for future automation of these environmental immunodetection strategies. An immunosensor is an analytical device consisting of an immobilized biological component in intimate contact with a transduction device that converts the immunorecognition event into a quantifiable electrical signal. When the Ab interacts specifically with the antigen (Ag), the generated physicochemical changes are sensed, in this case, electronically.

The aim of this chapter is to provide an overview of the potential of IAs as environmental analytical methods. Immunosensors will only be briefly mentioned, since more

information is provided in Chapter 22. We will describe the types and components of IAs (Sections 7.2 and 7.3) as well as the steps involved in their development and the criteria used for their developpoment (Sections 7.4–7.7). Finally, Section 7.8 wiil discuss aspects related to the validation of immunochemical techniques.

7.2 TYPES OF IMMUNOASSAYS

From all types of IAs, we can mainly distinguish between competitive versus non-competitive IAs and homogeneous versus heterogeneous IAs [18–24]. In the homogeneous assays, there is no separation between the free and bound phase before the detection step. Usually, the binding of the Ag to the Ab or vice versa modulates the activity of the reporter enzyme or label. The homogeneous assays are very suitable for monitoring processes due to their shorter analysis time compared to the heterogeneous assays; however, inconveniences such as matrix effects and insufficient sensitivity are often observed. Moreover, not all the Ags, especially low molecular weight Ags, are able to modulate the activity of the enzyme, which explains the reduced impact of these assays in the environmental field [7,25–27].

Heterogeneous formats have found a broader application to many fields and also to different kinds of analytes. One of the immunoreactants is bound to a solid support making possible the separation between the bound and free phases without the need of modulating the activity of the label. When the analyte is a large molecule with two or more epitopes, the non-competitive IAs are the most common and easy to develop. For example, the immobilized Ag is recognized by the Ab and the immunoreaction is detected by a second labeled Ab. In another configuration, an excess of labeled Ab is used to detect the Ag captured by another Ab bound to the solid surface. This format is very popular and the assays are known as sandwich-type assays. Restrictions of this configuration are that the Ag must have epitopes and that usually both Abs should have their specificity directed to a different one.

However, most of the environmental pollutants are small molecules that cannot simultaneously interact with two Abs or be immobilized directly on a solid phase. In those cases competitive IAs should be employed. Non-competitive IAs have nevertheless been used for the detection of soil-bound pesticides The general strategy of competitive assays is based on the competition of the free Ag with a fixed amount of labeled Ag (Ag*) for a fixed and limiting amount (low concentration) of Ab. At the end of the reaction the amount of labeled Ag and subsequently the free Ag is determined. The most usual configurations are shown in Fig. 7.1 and are briefly described below; however, readers are referred to other publications for a more extensive description of the different types of IA formats [28].

7.2.1 Direct competitive assay

Ab coating format. An equilibrium is established between the Ab bound to the solid surface (either directly or through orientating reagents such as antibodies generated against immunoglobulins (anti-IgG) or protein A [29]), the analyte and the analyte-enzyme tracer which are in solution. After the main incubation step, the unbound reagents

are washed away and the amount of label bound to the solid phase by the Ab is measured. A decrease in the signal is directly proportional to the amount of analyte present.

Ag coating format. This is based on the competition between the immobilized Ag (or surface derivatized analyte) and the analyte for a fixed small amount of labeled Ab.

7.2.2 Indirect competitive assay

This works under the same principle as the Ag coating format, but the concentration of the analyte is measured in this case indirectly by the quantitation of bound Ab with a second labeled Ab.

7.2.3 Labels

The labels used to quantify the immunoreaction can be of different nature. Enzymes are the most common labels used in IAs for environmental analysis. Enzymes frequently used are horseradish peroxidase (HRP), glucose oxidase (GO), alkaline phosphatase (AP) and β-galactosidase (βG). These specific enzymes react with a convenient substrate, producing a chromogen often absorbing in the visible region. Their extremely high catalytic power amplifies the signal caused by the immunoreaction, increasing the sensitivity of the IAs. EIAs consist, thus, of a two-pronged strategy: the reaction between the immunoreactants (Ab with the corresponding Ag) and the detection of that reaction using enzymes coupled to the reactants, as indicators or labels. EIA techniques can be divided in two main groups: enzyme multiplied immunoassay techniques (EMIT) and enzyme-linked immunosorbent assay (ELISA). The first one is a competitive homogeneous IA. Few EMITs have been described for environmental monitoring of pollutants. As an example a homogenous IA has recently been developed for the pyrethroid permethrin reaching a detection limit of about 2–5 ng ml^{-1} by modulating the activity of the enzyme amylase [25]. ELISAs are the most popular EIAs used on environmental analysis (see Table 7.1). They are competitive heterogeneous assays which can be performed immobilizing one of the reagents on a variety of solid supports (tubes, microtiter plates, plastic-baked nitrocellulose membranes, magnetic particles, etc.). Because of their potential for processing simultaneously many samples, ELISAs have become popular for the rapid screening of organic pollutants in the environment, as it is shown by the numerous reviews and articles [20,21,30–38]. Examples of ELISAs for all types of pesticides (organophosphorus [39–42], carbamates [12,43–45], triazines [46–51]) and some industrial contaminants (PAHs [52], PCBs [53], phenols [54–56]) can be found in the literature.

In RIA the emission of radiation of an isotope is used to detect the immunoreaction. Radioligands, especially those that emit gamma radiation, can be rapidly, conveniently, and sensitive counted. One of the most commonly used isotopes is ^{125}I, which provides high specific radioactivity in comparison to tritium or ^{14}C-labeled ligands and also because it does not require using scintillation fluids. Picogram-level determinations can be usually reached. These kind of IAs were the first developed assays for environmental monitoring [2,57]. Thus, RIAs have been described for *S*-bioalletrin [58], PCDDs [59], parathion [60], PCBs [61], paraquat [62], benomyl [63], etc. Nevertheless, because of the precautions that have to be taken when manipulating radioactive substances, the generation of radioactive residues and the adverse health effects produced, others types of labeling substances have gradually replaced radioactive labels.

Fluoro immunoassay (FIA) and chemiluminescence immunoassay (CLIA) use fluorescent labels such as fluorescein, rhodamine or rare earth quelates such as Eu(III), Tb(III), Sm(III) chelates and chemiluminescent labels such as luminol [64,65]. The former are faster and have more precision than RIA [35], but the latter present a considerable loss of the luminescent quantum yield of the label after the coupling reaction. Another inconvenience is the presence of matrix components catalyzing the luminescent reaction. In the case of fluorescent labels, the sensitivity may be limited by the background noise of some samples [66]. To enhance detection in most FIAs for pesticides, the fluorophore is generated enzymatically rather than using them directly as labels and therefore can also be considered as EIAs. FIAs have often been adapted to flow-immunoassay sytems (see Section 7.2.2). FIAs and CLIAs have been described for many analytes such as dichloprop [67], 2,4-dichlorphenoxyacetic acid (2,4-D) [27], diclofop-methyl [68], triazines [65,69–71], triasulfuron [72], etc.

Liposome-based IAs use liposomes containing usually water-soluble fluorescent or electroactive molecules [73]. These liposomes are used to label either the Ag or the Ab depending on the IA format used (see Fig. 7.1). For example, in one of these assays [74]; a solution containing a mixture of alachlor and alachlor tagged dye-containing liposomes are allow to migrate through an anti-alachlor Ab zone, on a plastic-backed nitrocellulose strip, where competitive binding occurs. Unbound liposomes continue migration to a liposome capture zone, where they are quantified either visually or by densitometry. The amount of liposome-entrapped dye that is measured in this zone is directly proportional to alachlor concentration in the sample. A liposome immunoassay was developed for PCBs analysis in a nitrocellulose strip with a detection limit of 2.6 pmol [75] (see Fig. 7.2). The assay takes place in just 23 min and the measurement of color intensity is carried out visually or with a desktop scanner. Similarly, this kind of label has frequently been used on immunosensor configurations by combining it with electrochemical or optical transducer systems [76,77].

Other perspectives include the use of organometallic markers such as $Cr(Co)_3$, $Mn(CO)_3$, $Co_2(CO)_6$, etc., and their detection by Fourier transform infrared spectrometry (FT-IR) [78,79]. The differences encountered on the spectra profiles of these labels prompts to the possibility of developing multianalyte metalloimmunoassay procedures. Similarly, it has been suggested recently to use mass spectromety as a detecting system [80]. The approach of the mass spectrometry IAs would consist in the microscale immunoaffinity capture of target Ags followed by mass-specific identification and quantitation using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF–MS). Mass spectrometric detection of Ags is unambiguous, as Ag signals are observed at characteristic mass-to-charge values in the mass spectrum. Moreover, another important aspect of such mass-specific detection is the ability to use a single assay to screen biological systems for the presence of multiple, mass-resolved Ags. This strategy has been used for the detection of mycotoxins.

7.2.4 Other types of immunoassay configurations

The solid support used to immobilize the immunoreagents determines some of the formats used. IAs are mainly carried out in 96-well polystyrene, polyethylene, polypropylene or polyvinyl microtiter plates. Polystyrene tubes are also quite popular as well as

TABLE 7.1

FEATURES OF SOME OF THE IAs REPORTED FOR DIFFERENT FAMILIES OF ENVIRONMENTAL POLLUTANTS

Analyte	Type[a]	Format[b]	Ab[c]	IC_{50}[d]	LDD[d]	Matrices	Ref.
Carbamates							
Carbaryl	ELISA	I, MtP	MAb	0.058	0.010	Buffer	[43]
	ELISA	I, MtP	PAb	2–5	0.2	Buffer	[201]
		D, MtP		0.4–0.6	0.05	Water, milk, honey, urine, soil	
	FIS	nr	MAb	0.346	0.029	Buffer, honey, water	[237]
Carbofuran	ELISA	MgP	PAb	0.82	0.056	Water	[197]
				nr[f]	5.6	Soil	
	ELISA	I,MtP	MAb	1.8	0.2	Buffer, Fruit juices	[226]
Methiocarb	ELISA	D, MtP	MAb	0.02–0.20	nr	Buffer	[44]
		I, MtP					
		DD, MtP					
Molinate	ELISA	D, MtP	PAb	97.6	15.0	Water	[12]
Chloroacetanilides							
Alachlor	ELISA	I, MtP	PAb	0.5	0.2	Water	[263]
Amidochlor	ELISA	I, MtP	PAb	2.0	nr	Buffer	[264]
Butachlor	ELISA	I, MtP	PAb	7.0	nr	Water	[264]
Clomazone	ELISA	I, MtP	PAb	12.0	0.01	Soil	[265]
Metalaxyl	ELISA	I, MtP	PAb	0.50	nr	Buffer, Food	[266]
Metolachlor	ELISA	I, MtP	MAb	0.6	0.05	Water	[267]
		D, MtP		1.0	0.1		
	ELISA	I, MtP	PAb	6.0	nr	Buffer	[264]
Nitroaromatic							
2,4,6-TNT[e]	ELISA	D, MtP	PAb	210	2.0	Buffer, soil	[268]
2,4,6-TNT	ELISA	D, MtP	PAb	9.9	0.04	Water	[269]
Organochlorine							
Chlordane	FOB*	nr	MAb	nr	0.41	Buffer	[270]

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2,4-D[e]	ELISA	I, MtP	MAb	0.8	nr	Buffer	[271]
2,4-D	ELISA	D, MtP	PAb	4.0	0.3	Buffer	[272]
2,4-D	ELISA	I, MtP	PAb	nr	0.05	Buffer	[269]
DDA[e]	ELISA	D, MtP	PAb	0.8	nr	Buffer	[273]
DDE[e]	ELISA	D, MtP	PAb	9.0	nr	Buffer	[273]
DDT[e]	ELISA	I, MtP	MAb	0.74	nr	Buffer	[152]
DDT	ELISA	D, MtP	PAb	13.0	nr	River water, soil, tomato puree, apple puree, milk	[273]
DDT + DDE	ELISA	D, MtP	PAb	2.0	0.3	River water, soil, tomato puree, apple puree, milk	[273]
Dicofol	ELISA	D, MtP	PAb	3.0	nr	Buffer	[273]
Diclofop-methyl	ELISA	D, TT	PAb	nr	23.0	Soil, milk, urine, sugar beets, soybeans, grains of wheat and wheat shoots saMTPles	[68]
	FIA	nr	PAb	nr	45.0		
Endosulfan	ELISA	D, MtP	PAb	1.6	0.2	Water, soil	[141]
Fluroxypyr	ELISA	D, MtP	PAb	1.6	0.1	Water	[274]
				nr	5.0	Soil	
Picloram	ELISA	I, MtP	PAb	140	5.0	Water, soil, plants, urine	[275]
	ELISA	I, MtP	MAb	10.0	1.0		
	RIA		PAb	nr	50.0	River water, urine	[11]
Triclopyr	ELISA	D, MtP	PAb	1.7	0.1	Water	[274]
				nr	1.0	Soil	
Organophosphorous							
Azinphos-methyl	ELISA	D, MtP	MAb	3.0	0.4	Buffer	[229,276]
		I, MtP		8.88	100	Orange and apple juices	
	ELISA	I, MtP	MAb	1.0	0.04	Buffer	[277]
Chlorpyrifos-ethyl	ELISA	I, MtP	MAb	0.28	nr	River, well and irrigation waters	[153]
		D, MtP		0.35	nr		
	ELISA	D, TT	PAb	0.8–1.6	0.15	Irrigation, drainage and soil saMTPles	[222]
		D, MtP		0.2–0.6	0.05–0.07		
	ELISA	I, MtP	PAb	150	25.0	Wheat	[278]
Chlorpyrifos-methyl	ELISA	D, MtP	MAb	29	1.0	Wheat grain and flour milling fractions	[150]

TABLE 7.1 (*continued*)

Analyte	Type[a]	Format[b]	Ab[c]	IC$_{50}$[d]	LDD[d]	Matrices	Ref.
Diazinon	ELISA	D, MtP	PAb	0.4	0.05	Surface water	[202]
		I, MtP		0.4	0.02	Well water	
		D, TT		nr	0.05	Fruit juice	
Fenitrothion	ELISA	I, MtP	PAb	90.0	1.0	Wheat	[149]
		I, MtP	MAb	70.0	4.0		
		D, MtP	PAb	23.0	2.0		
		D, MtP	MAb	28.0	3.0		
Parathion	ELISA	I, MtP	MAb	119	16.0	Rice, orange	[122]
	ELISA	I, MtP	RAb	116	23.0		
	FOI	nr	PAb	nr	1.0	Buffer	[279]
Pirimiphos-methyl	ELISA	D, MtP	MAb	2.1	0.2	Buffer	[150]
				nr	30.0	Wheat grain and flour milling fractions	
PAHs							
Pyrene	ELISA	I, MtP	PAb	0.58	0.04	Water	[280]
Phenanthrene	ELISA	D, MgP	nr	16.5	0.70	Buffer	[52]
				nr	2.66	Water	
				nr	200	Soil	
PCBs							
3,4,3′,4′-Tetrachlorobiphenyl	ELISA	D, MgP	MAb	1.0	nr	Buffer	[281]
		D, MtP		0.9	0.2		
Aroclor 1248.	ELISA	D, MgP	nr	1000	nr	Soil	[220]
	ELISA	I, MtP	PAb	22.0	1.34	Water, soil, SRM (soil), sediments, paper pulp	[53]
Aroclor 1254	ELISA	D, TT	PAb	5000	nr	Soil	[221]
Phenolic compounds							
2,4-DNP[e]	ELISA	I, MtP	nr	1.84–0.036	nr	Buffer	[282]
	ELISA	I, MtP	PAb	2.03	0.26	Buffer, river water	[56]
	BIA	nr	PAb	2.05	nr	Buffer	[283]
1-Naphthol	ELISA	I, MtP	PAb	72.0	8.0	Buffer, urine, soil	[136]

4-NP[e]	ELISA	I, MtP	PAb	8.21	0.3	Buffer	[163]
4-NP	ELISA	I, MtP	PAb	9.32	0.62	Buffer, river water	[55]
PCP[e]	ELISA	I, MtP	MAb	nr	30–40	Buffer	[54]
	ELISA	D, TT	PAb	500	nr	Soil	[284]

Phenylureas

BAY SIR 8514	ELISA	I, PC	PAb	9.0	nr	Water	[146]
Chloroturon	ELISA	D, MtP	PAb	nr	0.015	Water	[285]
Chlorsulfuron	ELISA	I, MtP	PAb	nr	0.4	Food	[286]
Diflubenzuron	ELISA	I, PC	PAb	3.0	nr	Water	[146]
Diuron	ELISA	D, MtP	MAb	2.3	0.6	Buffer	[144]
	ELISA	I, MtP	PAb	1.6	nr	Foods	[145]
	ELISA	D, MtP	PAb	0.4	0.04	Buffer	[131]
	ELISA	D, MtP	PAb	0.12	0.01	Water	[287]
	FIIA	D	PAb	nr	0.02	Water	[288]
Isoproturon	ELISA	D, MtP	PAb	nr	0.03	Water	[289]
Linuron	ELISA	D, MtP	PAb	1.6	nr	Foods	[131]
	ELISA	D, MtP	PAb	0.8	0.08	Buffer	[131]
Monolinuron	ELISA	I, MtP	PAb	0.9	nr	Food	[145]
Monuron	ELISA	D, MtP	PAb	0.5	0.05	Buffer	[131]
Penfluron	ELISA	I, PC	PAb	6.8	nr	Water	[146]

Polychlorinated dibenzo-p-dioxins

TCDD[e]	ELISA	I, MtP	PAb	0.24	nr	Buffer	[203]
	ELISA	D, MgP	MAb	nr	0.1	Soil	[290]
		D, MtP		nr	0.025	Water	
	ELISA	I, MtP	MAb	0.005–0.03	nr	Buffer	[291]

Pyrethroids

Allethrin (S-bioallethrin isomer)	ELISA	I, MtP	MAb	46.0	1.0	Buffer	[292]
	ELISA	I, MtP	PAb	6.0	0.5	Buffer	[293]
Permethrin	ELISA	I, MtP	MAb	150	nr	Meat extract	[262]
	ELISA	D(CA), MtP	MAb	15.0	2.0	Grain, flour, methanolic extracts	[294]
		D, MtP		10.0	1.5		

TABLE 7.1 (*continued*)

Analyte	Type[a]	Format[b]	Ab[c]	IC$_{50}$[d]	LDD[d]	Matrices	Ref.
	ELISA	I, MtP	PAb	1.0	0.5	Buffer	[295]
	EMIT	MtP	PAb	8–10	2.0	Buffer	[25]
Bioresmethrin	ELISA	I, MtP	PAb	25.0	2	Wheat, barley	[292]
Deltamethrin (isomerized)	ELISA	D, MtP	PAb	2.0	0.2	Water, soil, grain	[134,143]
Phenoxybenzoic acid	ELISA	(CAIA)	MAb	100	nr	Buffer	[296]
Semi-volatile compounds							
Benzene, toluene, xylenes	ELISA	D, MtP	PAb	1700	210	Water	[297]
Sulfanylides							
Metosulam	ELISA	D, MtP	nr	2.800	0.3	Water, soil	[298]
Sulfonylurea							
Chlorsulfuron	ELISA	I, MtP	PAb	nr	0.1	Water, soil	[286]
Metsulfuron-methyl	ELISA	I, MtP	MAb	1.4	0.04	Water	[299]
Triasulfuron	ELISA	D, MCI	nr	0.480	0.05	Water, soil	[215]
	CLIA	I, MgP	MAb	nr	0.02	Water, soil	[72]
	ELISA	nr	PAb	nr	0.04	Water, soil	[300]
	ELISA	I, MtP	MAb	nr	0.1	Water, soil	[216]
Triazines							
Atrazine	ELISA	DD, MtP	MAb	0.24	0.03	Water, Soil	[46]
	ELISA	D, MtP	PAb	0.06	0.009	Water	[51]
	ELISA	D, TT	PAb	nr	0.5	Food	[301]
	ELISA	D, TT	PAb	0.4	0.1	Water, soil	[217,218]
	ELISA	D, MtP	PAb	nr	0.011	Fresh water	[302]
	ELISA	D, MtP	PAb	0.13	0.010	Water	[303]
	ELISA	DD, MtP	Ab	3.0	0.05	Water, soil	[304]
	FIIA	D	MAb	0.1	0.02	Water	[288]
Hydroxyatrazine	ELISA	I, MtP	PAb	nr	0.10	Water	[305]
Hydroxypropazine	ELISA	I, MtP	MAb	0.50	0.05	Water, Soil	[219]
Hydroxysimazine	ELISA	I, MtP	PAb	5.9	nr	Groundwater	[306]

Irgarol	ELISA	I, MtP	PAb	7.0	nr		Groundwater	[306]
Propazine	ELISA	D, MtP	PAb	nr	nr		Water	[307]
Simazine	ELISA	D, MtP	MAb	0.02	0.003		Groundwater	[308]
	ELISA	D, MtP	MAb	nr	0.01		Groundwater	[308]
Terbuthylazine	ELISA	DD, MtP	PAb	0.1	0.05		Groundwater	[309]
	ELISA	D, MtP	MAb	nr	0.004		Groundwater	[308]
	ELISA	DD, MtP	MAb	0.8	0.14		Water	[310]
	FIA	D, MtP	MAb	0.06	nr		Water	[69]
Terbutryn	ELISA	D, MtP	MAb	0.17	0.03		Water	[311]
Terbutryn	FIA	D, MtP	MAb	nr	0.1		Water	[71]
	ELISA	I, MtP	MAb	0.20	0.03		Water	[312]

[a] Type of IA: Bioimmunoassay (BIA), Capillary enzyme immunoassay, Chemiluminescence immunoassay (CLIA), Enzyme-linked immunosorbent assay (ELISA), Enzyme multiplied immunoassays techniques (EMIT), Flow immunoassay (FIA), Flow-injection immunoassay (FIIA), Flow immunosensor (FIS), Fiber-optic biosensor (FOB), Fiber-optic immunosensor (FOI), Radioimmunoassay (RIA).

[b] IA format: Direct assay (D), Direct assay double coating with goat anti-mouse or anti-rabbit (DD) Indirect (I), Direct coating antigen format D(CA), Test tubes (TT), Magnetic particles (MgP), Microtiter plates (MtP), Ab coating format/anti-idiotipic antibodies (CAIA), Magnetic particle-based automated chemiluminiscent IA (MCI), Polystyrene cuvettes (PC).

[c] Monoclonal (MAb), polyclonal (PAb) or recombinant antibodies (RA)

[d] Values expressed in μg l^{-1}.

[e] 2,4-D, 2,4-dichlorphenoxyacetic acid; DDA, 2,2-bis(p-chlorophenyl) acetic acid; DDE, 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethene; DDT, 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane; 2,4-DNP, 2,4-dinitrophenol; 4-NP, 4-nitrophenol; PCP, pentachlorophenol; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

[f] nr, not reported.

TABLE 7.2

SOME OF THE ELISAs COMMERCIALLY AVAILABLE FOR ENVIRONMENTAL ANALYSIS

Analytes	Type	Immob. reag.[a]	Range[b]	LDD[b]	IC$_{50}$[b]	Supplier	Ref.
Alachlor	MgP	Ab	0.05–5	0.05	0.98	SDI	[257]
Aldicarb	MtP	Ab	1	0.4	5	Immunosystems/SDI	–
	MgP	Ab	0.25–100	0.25	9.28	Ohmicron/SDI	[227]
Acetanilide	MgP	Ab	0.1–5	0.02	1.5	Immunosystems/SDI	–
Atrazine	MgP	Ab	0.05–5	0.046	0.72	Ohmicron/SDI	[250]
	MgP	Ab	0.015–1	0.015	0.22	Ohmicron/SDI	[210]
	TT	Ab	–	0.05	–	Immunosystems/SDI	–
Bioresmethrin	MtP	Ab	–	0.08	–	Immunosystems/SDI	–
Captan	MgP	Ab	10–3000	10	420	Ohmicron/SDI	–
Carbaryl	MgP	Ab	0.25–5	0.25	2.57	Ohmicron/SDI	–
Carbendazim/ Benomyl	MtP	Ab	0.4–10	0.1	2	Immunosystems/SDI	–
	MgP	Ab	0.1–5	0.1	1.6	Ohmicron/SDI	[254]
Carbofuran	TT	Ab	–	0.1	–	Immunosystems/SDI	–
	MgP	Ab	0.06–5	0.06	0.82	Ohmicron/SDI	[197]
Chlorothalonil	MgP	Ab	0.07–5	0.07	1.12	Ohmicron/SDI	[258]
Chlorsulfuron	MtP	Ab	0.04–0.8	0.04	0.22	Immunosystems/SDI	–
Chlorpyrifos	MgP	Ab	0.1–3	0.1	0.94	Ohmicron/SDI	[198]
	TT	Ab	–	100	–	Immunosystems/SDI	–
Cyanazine	MtP	Ab	0.25-	0.14	0.73	Immunosystems/SDI	–
	MgP	Ab	0.04–3	0.035	0.43	Ohmicron/SDI	–
2,4-D	TT	Ab	–	1.6	–	Immunosystems/SDI	–
	MtP	Ab	0.5–100	0.1	2.3	Immunosystems/SDI	–
	MgP	Ab	0.7–50	0.7	15	Ohmicron/SDI	–
Fenitrothion	TT	Ab	–	100	–	Immunosystems/SDI	–
	MtP	Ab	–	150	–	Immunosystems/SDI	–

Fenoprop	MgP	Ab	5–250	1.4	58	Ohmicron/SDI	—
Hexazinone	MtP	Ab	—	0.08	—	Immunosystems/SDI	—
Isoproturon	MtP	Ab	0.05–0.5	0.02	0.13	Immunosystems/SDI	—
	MtP	Ab	—	0.02	—	Immunosystems/SDI	—
Methomyl	MgP	Ab	0.45–15	0.45	4.15	Ohmicron/SDI	[259]
Methoprene	MtP	Ab	—	1000	—	Immunosystems/SDI	—
	MgP	Ab	0.05–5	0.05	0.85	Ohmicron/SDI	[253]
	MtP	Ab	—	0.07	—	Immunosystems/SDI	—
Metribuzin	MgP	Ab	—	0.04	—	Ohmicron/SDI	[260]
Metsulfuron	MtP	Ab	0.025–0.5	0.02	0.1	Immunosystems/SDI	—
Molinate	MtP	Ab	—	0.5	—	Immunosystems/SDI	—
Paraquat	MtP	Ab	—	0.006	—	Immunosystems/SDI	—
	MgP	Ab	0.20–0.5	0.020	0.30	Ohmicron/SDI	[261]
Parathion-methyl	MtP	Ab	0.04–0.4	0.03	0.3	Immunosystems/SDI	—
Pirimiphos	TT	Ab	—	200	—	Immunosystems/SDI	—
	MtP	Ab	—	50	—	Immunosystems/SDI	—
Procymidone	MgP	Ab	0.8–100	0.8	19	Ohmicron/SDI	—
Simazine	MgP	Ab	—	0.03	—	Ohmicron/SDI	—
Thiabendazole	MtP	Ab	0.25–4	0.25	—	Immunosystems/SDI	—
Triasulfuron	MtP	Ab	0.05–1	0.04	0.36	Immunosystems/SDI	[215]
Triazines	TT	Ab	—	0.053	—	Immunosystems/SDI	—
	MtP	Ab	0.05–20	0.02	0.25	Immunosystems/SDI	—
	MtP	Ab	0.01–0.5	0.01	0.08	Immunosystems/SDI	—
3,5,6-trichloro-2-pyridinol	MgP	Ab	0.25–6	0.25	2.31	Ohmicron/SDI	[151]
Diuron	MtP	Ab	—	0.02	—	Immunosystems/SDI	

[a] Immobilized reagent.

[b] Quantification range, IC_{50} and LDD are expressed in μg l^{-1}. TT, test tubes; MtP, microtiter plate; MgP, magnetic particles.

TABLE 7.3

DETECTABILITIES REPORTED IN DIFFERENT MATRICES OF SOME COMMERCIALLY AVAILABLE ELISAs FOR ENVIRONMENTAL ANALYSIS OF POLLUTANTS FROM INDUSTRIAL ORIGIN[a]

Analytes		Field Tests			Lab Screen			
		EnSys	Envirogard	Envirogard	EnSys	Water	Ohmicron	Ohmicron
		Soil	Soil	Water	Soil	Water	Soil	Water
TPH	Gasoline	10	7	60	10	–	4	0.4
	Diesel	15	14	0.1	15	–	13	1.3
	JP-4	15	12	0.1	–	–	27	2.7
	Kerosine	15	6	0.1	15	–	15	1.5
	Fuel oil #2	15	14	0.1	15	–	–	–
	Fuel oil #4	25	25	0.1	25	–	–	–
PAH		1	1	0.002	0.03	0.0009	0.2	0.0007
Carcinogenic PAH		–	–	–	–	–	0.004	0.00004
PCB		1	1	–	0.1	0.004	0.5	0.0002
PCB Wipe		10*	–	–	–	–	–	–
PCB Liquid Waste		–	–	–	–	5 (oil)	–	–
PCP		0.5	–	–	0.5	0.005	0.1	0.00006
DDT		–	0.2	0.02	–	–	–	–
Lindane		–	0.4	0.03	–	–	–	–
Toxaphene		–	0.2	0.02	–	–	–	–
Cyclodienes		–	0.01	0.0009	–	–	0.1	0.0006
Chlordane		–	0.014	0.0012	–	–	–	–
TNT		–	–	–	–	–	0.25	0.0007

[a] Concentrations of LDD expressed in mg l^{-1} for waters and in μg g^{-1} for soils, except * that is expressed as μg cm^{-2}.

TABLE 7.4

EPA IA METHODS USEFUL FOR THE SEPARATION, DETECTION AND QUANTITATION OF ORGANIC POLLUTANTS IN DIFFERENT ENVIRONMENTAL MATRICES

Method	Immunoassay	Matrix
4010A	PCP	Water and soil
4015	2,4-D	Water and soil
4016	2,4,5-T	Soil
4020	PCB	Soil and oil
4030	TPH	Soil
4035	PAH	Soil
4040	Toxaphene	Soil
4041	Chlordane	Soil
4042	DDT	Soil
4050	TNT	Water and soil
4051	RDX	Water and soil
4500	Mercury	Soil
4670	Triazines	Water[a]

[a] Submitted.

polystyrene, latex or polycarbonate beads. In this case, separation of free and bound reagents is performed by centrifugation. Magnetic beads are also frequently used and several IAs for pesticide determination are commercially available in this format (see Table 7.1) that uses a magnet for the separation step. Immunoreagents can also be immobilized covalently to amino- and carboxy-modified surfaces by standard coupling methods. IAs may as well be performed in membranes as dipsticks or immunofiltration assays. These assays are usually applied for field-testing and the analysis can be performed in a few minutes. The test principle is the same as for the microtiter plates but the reaction time is much shorter due to the high surface area of the membrane and the short distance between the reaction partners. The liposome immunomigration strip assays previously mentioned are examples of these kinds of assay [75,81]. Similar assays have also been described for triazines [82–85], mycotoxins [86,87], marine toxins [88], etc.

Polarization fluoroimmunoassay (PFIA) measures the increase of fluorescence polarization when a specific Ab binds a fluorophore-labeled hapten. Similarly, a decrease in the signal is observed when a free analyte competes with the labeled hapten for binding to the Ab. PFIAs has been described for several pesticides [89] such as simazine [90], atrazine [26], propazine [70], dichloprop [67], 2,4-D [27], etc.

Enzyme-linked immunofiltration assay (ELIFA), consists in coating the Abs on a membrane instead of a plastic surface. In this way, it has been claimed that detectability can be greater due to the major adsorbance of the proteins on the membrane. Furthermore, time analysis is shorter than in other IAs because the different solutions are sequentially dropped-on.

Flow injection immunoassays (FIIA) apply the characteristic unsegmented continuous flow from FIA techniques to automate IA procedures. This configuration improves test time and reliability by maintaining the same detectability. For review articles on FIIAs see [91–93]. FIIAs have been described for a variety of pollutants such as triazines [51,84,94–

A Direct Competitive ELISA

Coating Antigen

Analyte

IgG

IgG-HRP

B Indirect Competitive ELISA

AntiIgG-HRP

Enzyme Tracer

TMB Substrate
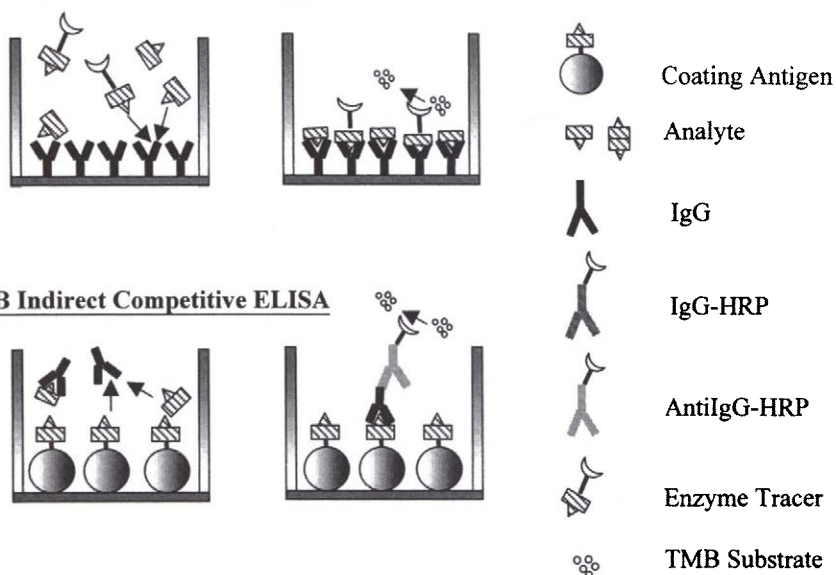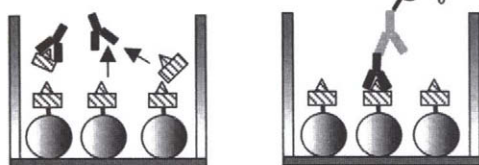
C Sandwich ELISA
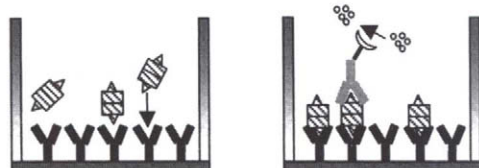
Fig. 7.1. Scheme of the heterogeneus IA configurations most commonly employed (see text for description). From Marcop et al. [313].

96], explosives [97], PCBs [98], etc. Using a similar concept, flow injection liposome immunoassays (FILIA) have been reported for the herbicide imazethaphyr [99].

7.3 ANTIBODIES

7.3.1 Chemical structure

Abs are the key reactives of the immunochemical techniques. Abs are polypeptides belonging to the immunoglobulin (IgG) family. The Abs used in most of the immuno-chemical techniques are IgGs formed during the humoral response of the immune system of the mammals when in contact with a foreign substance or Ag. The IgGs (MW 150 000 Da) are monomers of Igs formed by two pairs of polypeptidic chains interconnected by disulfide bonds (see Fig. 7.3). Two chains contain approximately 450 amino acid residues and are known as the heavy chains, contrary to the light chains with only 212 amino acid residues. Both light and heavy chains are divided into constant and variable regions. The constant region or crystallized fraction (Fc) has the same amino acid sequence in all the Iid
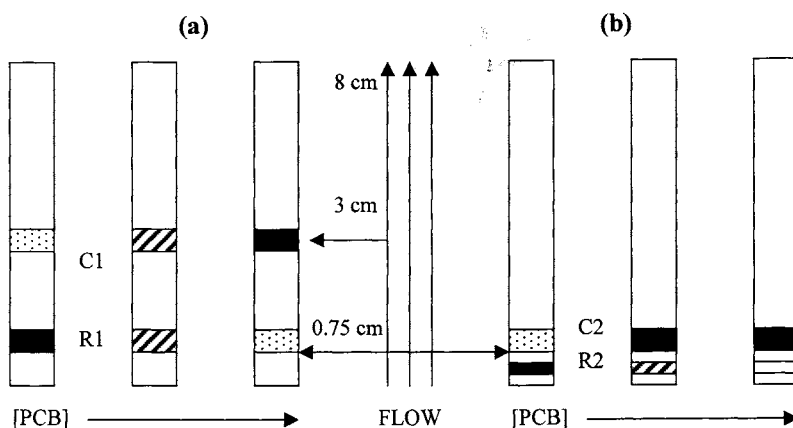
Fig. 7.2. (a) Liposome immunocompetition assay (LIC); R1, liposome/PCB competition zone. (b) Liposome immunoaggregation assay (LIA); R2, liposome-antibody aggregation zone. C1, C2, anti-biotin capture zones. Agreed to and accepted by ACS Copyright Office [75].

sequence of this region, unique on each IgG produced by a single B-cell clone. This amino acid sequence and the spatial conformation of the binding site determine the specificity of the molecule. The interaction between Ab and Ag is reversible and it is stabilized by electrostatic forces, hydrogen bonds, hydrophobic and Van der Waals interactions.

7.3.2 Antibody production

Several types of Abs are used for immunochemical techniques. The most common Abs used on IAs for environmental analyses are polyclonal antibodies (PAb) and monoclonal
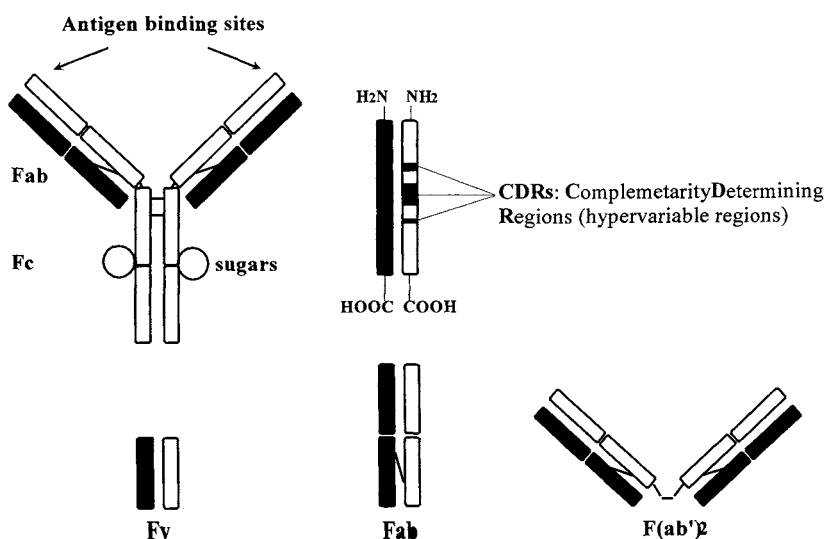
Fig. 7.3. Structure of immunoglobulin G (IgG) molecules.

antibodies (MAb) (see Sections 7.3.2.1 and 7.3.2.2). The preparation of MAbs by in vitro immunization [100–102], using lymphocytes in a culture media, has arisen as an interesting approach. Thus, studies performed using transgenic plants as living bioreactors for the production of important biomolecules, such as MAbs, could be promising [103,104]. Finally, DNA technology is an exciting strategy for producing MAbs or fragments [104–106]. Although few IAs based on recombinant DNA antibodies (RAb) have been described, the potential advantages of using this approach are widespread in the near future.

In addition to the recombinant DNA approach, Ab fragments keeping the Fab region have been obtained by using specific enzymes (pepsin: $F(ab')_2$ and Fc fragments; papain: 2 Fab and 1 Fc fragment). Paralog peptides (MW 1000–2000 Da) of the CDRs can also be produced chemically by solid-phase synthesis [107]. Finally, Ab fragments can also be obtained by using reducing agents that dissociate the disulfide bonds to generate free sulfhydryl groups used on some immobilization procedures. As an example, mercaptoethylamine reduces the disulfur bond of the hinge region without dissociating the heavy and light chains [108].

7.3.2.1 Polyclonal antibodies

PAbs are obtained by immunizing mammals with the target Ag. The more common animals immunized are rabbits, mainly when large volumes of serum are not required, but many other species can be used such as goats, pigs, sheep or cows. Immunization protocols are well established [109]. Intradermal injections of the immunogen mixed with an adjuvant, to enhance the immune response, are carried out each 4 or 5 weeks. Generally, after 3–6 injections no increase in the Ab titer is observed. The collected antiserum can be used

TABLE 7.5

ADVANTAGES AND LIMITATIONS OF IAs

Advantages	Limitations
Wide applicability	Haptens can be difficult to synthesize
Sensitive and specific	Can be vulnerable to cross-reacting compounds
Suitable to field use	Possibility of non-specific interferences
Usually reduced sample preparation	Requires independent confirmation
Easy of use	Inappropiate for small sample loads or multi-residue determinations
Rapid	High development costs
High sample throughput	Lack of acceptance, conservative attitudes
Simultaneous analysis of multiple samples	
Low cost of analysis	
For certain polar compounds, IAs are the choice	

directly as immunoreagent, although in some cases IgGs are purified, often by precipitation with saline solutions or by affinity chromatography.

The immune response is a complex process where molecular recognition and communication episodes between specialized cells of the immune system take place thorough certain mediators to produce a cascade of events leading to the multiplication of specific clones of B-cells in the serum. In the immune system, a single B-cell produces a single type of IgG, which recognizes a little part of the Ag molecule. The antiserum contains a heterogeneous mixture of IgGs that recognize the global structure of the Ag with high specificity and sensitivity. For more extensive information on the mechanisms involved in the immune response, the reader is addressed to other documents [110–113]. However, it is important to note that the sequence of molecular recognition events involved in the immune response determines that Ags below certain molecular size are not able to trigger an immune response.

Many environmental pollutants are organic molecules with molecular weights below 1000 Da and consequently when they are administered to animals do not elicit antibodies. To raise Abs to them, it is necessary to chemically synthesize an appropriate hapten (see Section 7.4) and to couple it to a carrier, usually a protein. The avidity and specificity of the antiserum is determined by the chemical structure of the immunoconjugate (see Section 7.4.1.1), the immunization schedule, and also the particular immune system of the animal used. For this reason, it is recommended to immunize two or three animals with each Ag. The variability of the PAbs features from one animal to each other can be a limitation when a constant supply of identical antisera is required, for example, for commercial purposes.

7.3.2.2 Monoclonal antibodies

In contrast with PAbs, MAbs contain a unique defined IgG molecule that is produced by a single B-cell clone. The production of MAbs is well documented [104,114,115]. In this case, the mammals of choice are mice. After immunization, the mouse spleen is removed, and the spleen cells are fused with tumorgenic B-lymphocytes as myeloma cells for its immortalization. After fusing, the mixture is divided into many culture wells and allowed to growth. The presence of specific Abs in each well is tested, generally using a competitive ELISA, and the positive wells are further cloned. The operation is repeated until a B-cell clone producing Abs with the desired properties is isolated. The screening process may become tedious and time-consuming, increasing thus the cost of Ab production. MAbs production presents the advantage that, theoretically, it provides an unlimited amount of Abs with identical affinity for the Ag. Another advantage is the possibility to screen for those clones of Abs with the desired pattern of specificity. However, the widespread idea that MAbs provide more sensitive and selective IAs than PAbs is not true. Therefore, before addressing the Abs preparation, one should balance the cost of the process regarding the potential applicability of these Abs.

7.3.2.3 Recombinant DNA antibodies

This approach for obtaining Abs consists on the isolation of the genes encoding the

desired polypeptidic fragment from the Ab and their introduction into an expression-vector system (i.e. *Escherichia coli*). In general, the whole process would include the isolation of messenger ribonucleic acid (mRNA) from hybridoma, spleen cells or lymphocytes, synthesis of complementary deoxyribonucleic acid (cDNA) by reverse transcriptase, amplification of the RNA–DNA hybrid by polymer chain reaction (PCR) using suitable primers, ligation of cDNAs obtained in a bacterial plasmid vector, transformation of competent host cells and expression and screening for desired Ab fragments [116–120].

The advances achieved during the last years in the field of recombinant DNA technology, and the progressively deeper knowledge of the molecular structure of Abs and their interaction mechanism with the Ag, have allowed the production of Ab libraries generated by this technique. Only five to ten amino acid residues of each chain from the Fab region form the binding pocked of the Ab. The DNA technology would allow receptor design for each particular Ag, modeling a priori their size and composition, meaning the characteristics of the active site.

Although at the moment, very few recombinant Abs (RAbs) fragments for environmental contaminants have been described (triazines [118,120,121], parathion [122], dioxin [123], etc.), the economic factor is one of the advantages of this technique over the conventional methods of obtaining PAbs and MAbs. PAbs are easy to obtain but they are limited by the batch production; in contrast, MAbs are expensive to maintain and screen. Recombinant DNA libraries would allow a cost-effective production of Ab, moreover on a high scale.

7.4 HAPTEN DESIGN

This is the most crucial step on the development of an immunochemical technique for pesticides or other low molecular weight environmental pollutants. Specificity and selectivity of an immunochemical technique are mainly determined by the Ab [124–130] and the chemical structure of the competitor used as CA or enzyme tracer. Many examples in the literature prove that an appropriate hapten design determines the features of the resulting antibodies. A *hapten* is a molecule analogous to the target analyte and properly functionalized to allow covalent attachment to a carrier compound.

7.4.1 Effect of the chemical structure of the immunizing hapten

Immunizing hapten design is the key step in the development of IAs. As mentioned above, their selectivity and detectability are mainly determined by the quality of the resulting Abs. The immunizing hapten should represent a near perfect mimic of the target molecule in chemical structure, spatial conformation, electronic distribution and hydrophobic properties. However, sometimes characteristic portions of the molecule are sufficient to generate valuable antibodies. It is advisable to avoid modification of the immunogenic groups in the target molecule and/or introducing new ones, as this supposes an alteration of its structural, geometrical and electronic properties, and consequently a reduction of the sites for potential molecular recognition. Nowadays, it is possible to make theoretical calculations and computer models in order to predict the more appropriate chemical structure of the hapten [139–142].

Examples found in the literature demonstrate that the chemical function used for the

conjugation should be separated of the most important moiety of the target analyte by a spacer arm in order to avoid carrier hinderance. A 3–6 atom spacer length has often been considered as optimum size for the spacer [44,46,131–134], although not many exhaustive studies have addressed this point. Moreover, IAs have been described when Abs had been raised using haptens with shorter spacer arms [135,136]. The spacer arm should preferably replace a carbon hydrogen bond. Ideally, a spacer arm should be a chain of methylenes terminated by a functional group. The presence of bulky or other functional groups or heteroatoms may lead to the generation of antibodies versus the spacer arm and consequently to a poor recognition of the free analyte in the competitive ELISA [137,138].

7.4.1.1 Effect on the IA specificity

Different criteria have to be considered when analyte-specific Abs are desired. Thus, the exposure of the most characteristic part of the target compound should be maximized, attaching the spacer arm to the appropriate place. On the other hand, if a class-analyte assay is intended, the common part of the molecules has to be chosen for maximal exposure to the immune system. By using this criterion, Skerritt and Lee [39] obtained Abs and IAs showing different specificities against pyrethroids (see Fig. 7.4a). When the attachment took place in site Y, the characteristic groups of each pyrethroid molecule were exposed to the immune system and analyte-specific Abs were obtained. Similarly, class-specific Abs were obtained by attaching the spacer arm by site X thus maximizing the exposure of the phenoxybenzyl moiety which is common to several pyrethroid insecticides. Haptens with the linker placed in the middle of the molecule (site Z) were also synthesized by Lee et al. [134,143], and the resulting assays compared to those obtained from haptens prepared by site X and Y were the most sensitive (see Table 7.6). In this case a high recognition of the dihalovinylcyclopropane moieties was observed, suggesting that the Ab specificity may be directed toward this portion of the molecule rather than the aromatic groups. This fact was explained by the authors as a result of the greater distance existing between the 2'-vinyl carbon of the cyclopropane moiety to the coupling point than the C3' of the phenoxybenzylgroup.

Goodrow et al. [129] studied three possible handle attachment sites for haptens of arylurea herbicides monuron and linuron (see Fig. 7.4b). The preparation of a hapten with a spacer arm in site A is synthetically easier than the others, but also the resulting molecule mimics the target analyte very well. The linker hardly modifies its properties while keeping the aromatic ring far enough from the attachment site without altering the urea functional group. The features of the developed immunochemical assays have confirmed the predicted advantage of this hapten (see Table 7.7) [131,144]. Newsome and Collins [145] prepared immunizing haptens by introducing a linker in site C. The resulting polyclonal antisera showed a very poor specificity since many other chemical structures containing the dimethyl urea moiety were recognized in the assay. Finally, site B immunizing haptens probably produced important variations of the electronic and hydrophobic characteristics of the molecule. Additionally, the possibility of establishing hydrogen bonds in this position of the molecule has been altered. These haptens had been used as competitors by other authors [146], observing an important decrease of their recognition by the Ab.

7.4.1.2 Effect on the IA detectability

Knowledge on which are the most important groups of the molecule that can participate establishing non-covalent interactions with the Ab is important in order to obtain high-affinity antibodies. Low-affinity antibodies are expected to render IAs with low detectability. Thus, in our group, Ballesteros et al. [139] analyzed the potential participation to establish bonds with the Ab of the most important functional groups of the Irgarol 1051 molecule (see Fig. 7.4c). Three haptens differing in the attachment site of the linker were synthesized and the resulting Abs were screened to develop competitive ELISA. Computer molecular modeling studies were used to explain the differences encountered between the different immunizing haptens. This study demonstrated the prevalence in this case of non-covalent hydrophobic interactions stabilizing the analyte–Ab immunocomplex. Thus, the immunizing haptens (linkers in sites H and G) keeping the *tert*-butyl group rendered several IAs with inhibition concentration at 50% of B/B_0 (IC$_{50}$) below 0.3 μg l^{-1}. In contrast, the hapten lacking this group gave IAs with IC$_{50}$s above 1.0 μg l^{-1} (linker in site F). Another example is the case of the development of an immnoassay for the herbicide bromacil. Antibodies were generated by using haptens having the spacer arm in two different positions: through a carbon atom or blocking an amino group of the uracil ring. In this case the conversion of a secondary amine to a tertiary one reduced the possibilities of establishing non-covalent interactions with the Ab and thus, these antibodies rendered an IA showing lower detectability [147]. The same explanation can be given for the difference in sensitivity encountered with the IAs developed for urea herbicides [131,144] (see above). Antibodies obtained by coupling fenitrothion to the carrier protein through the nitro group led to assays with very poor detectability while the best assay used antibodies raised to a hapten coupled through the thiophosphate group, which suggests participation of the nitro group in stabilizing the immunocomplex [148–150]. Oubiña et al. (unpublished results) failed to obtain a competitive IA to determine 4-NP when using Abs that had been raised from a hapten blocking the phenolic group of the molecule.

7.4.2 Effect of the chemical structure of the competitor

Competitors are those haptens used to prepare coating antigens (CAs) and enzyme tracers. Contrary to the immunizing haptens, requirements regarding similiarities with the target are not so strict. In fact, there has often been claimed that certain heterology may favor assay sensitivity [44,47,131,151]. In this way, the equilibrium constant defining the formation of the immunocomplex competitor–Ab would be lower than the one directing the reaction between the analyte and the Ab. Optimal heterologous system are usually accomplished by the screening behavior of several haptens coupled to enzymes or proteins. These haptens may have different degrees of heterology depending on the variation of the chemical structure and the length and/or position of the spacer arm.

7.4.2.1 Effect on the IA detectability

Despite the heterology concept above mentioned, its application is not always general. Thus, Galve et al. [142] obtained a good assay for trichlorophenol by employing a homologous enzyme tracer. However, Abad et al. [43] improved the detectability of an ELISA
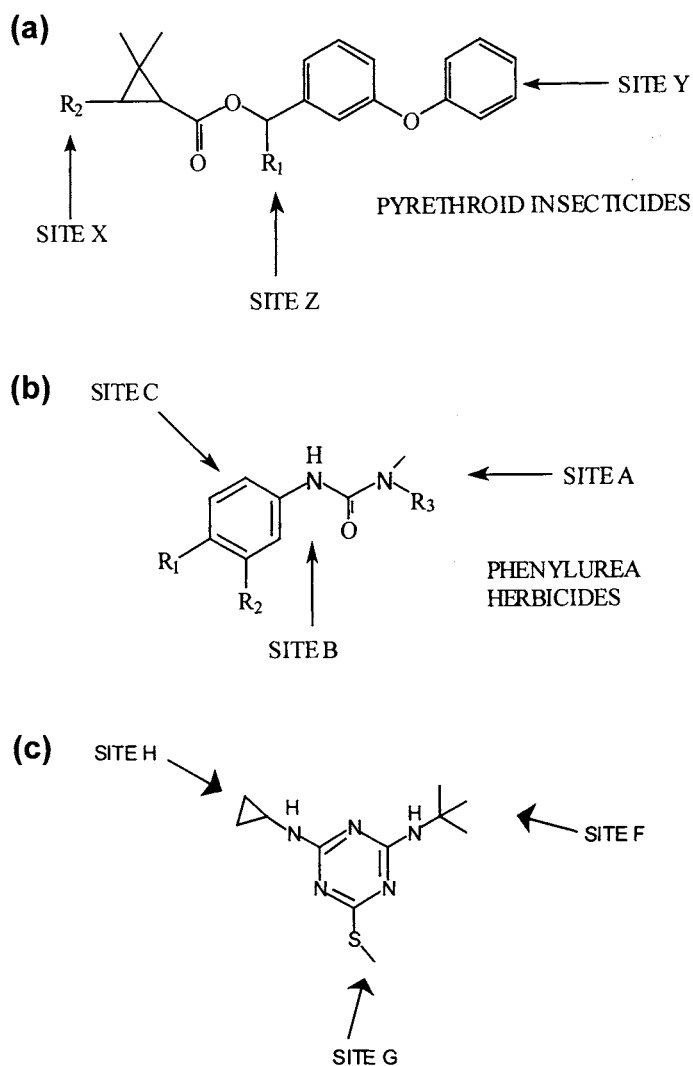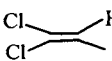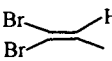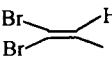
Fig. 7.4. Possible attachment sites to design immunizing haptens for different pesticides. (a) Pyrethroid insecticides, (b) phenylurea herbicides and (c) Irgarol 1051.

for carbaryl by using a heterologous hapten conjugate. But the same authors [152] produced MAbs to 1,1,1-(trichloro)-2,2-bis(p-chlorophenyl) ethane (DDT) where that heterology approach did not improve the limit of detection of the assay. One explanation could be that in the first case, only slight modifications were made on the length and position of linker while on the second one, changes were more severe since only a part of the total structure of the molecule was employed as competitor. Against that, a greater detectability was obtained on an IA for chlorpyrifos developed by Manclús and Montoya [153] by employing heterologous conjugates consisting in just a part of the complete analyte chemical structure. Aside of all these contradictory results, our experience

TABLE 7.6

LEAST DETECTABLE DOSE (LDD) AND PERCENTAGE OF CROSS-REACTIVITY (CR) OF DIFFERENT PYRETHROID INSECTICIDE IAs[a]

Pyrethroids	R_1	R_2	Attachment site[b]	LLD ($\mu g\ l^{-1}$)	%CR	Ref.
Permethrin	H	Cl, Cl (H)	Site X	1.50	1–10	[262]
Deltamethrin	H	Br, Br (H)	Site Y	20.0[a]	–	[125]
				0.40		
Deltamethrin	CN	Br, Br (H)	Site Z	5.0[a]	–	[125]
				0.20	–	

[a] The IAs used different sources of Abs raised against haptens with the linker placed on different sites.
[b] See Fig. 7.4 for chemical structures of the haptens and positions of the attachment sites.

suggests that high-affinity antibodies may be able to render excellent assays even under homologous conditions while the detectability of IAs using Abs with low affinity for the analyte may be increased by using heterologous competitors. Thus, from the three immunizing haptens evaluated to raise Abs against Irgarol 1051, we observed that those raised against hapten with the linker in sites H and G afforded IAs with high detectability using homologous or quasi-homologous competitors. In contrast, hapten with the linker in site F only afforded acceptable assays when the chemichal structure differed to a greater extent from that of the analyte [139] (see Fig. 7.4c and Tables 7.8 and 7.9).

7.4.2.2 Effect on the IA selectivity

Some authors claim that the chemical structure of the immunizing hapten is the main fact influencing IA specificity, while the chemical structure of the competitor mainly

TABLE 7.7
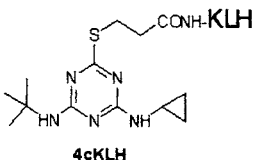
DETECTABILITY OF PHENYLUREA HERBICIDE IAs[a]

Phenylureas	R_1	R_2	R_3	Attachment site[b]	IC_{50} ($\mu g\ l^{-1}$)	Ref.
Diuron	Cl	Cl	CH_3	Site A	2.30	[144]
				Site B	n.c.	
Monuron	Cl	H	CH_3	Site A	0.50	[131]
Diuron	Cl	Cl	CH_3	Site C	16.0	[145]

[a] The IAs used distinct sources of Abs raised against different haptens.
[b] See Fig. 7.4 for chemical structures of the haptens and positions of the attachment sites. n.c., no competition.

Table 7.8

EFFECT OF THE CHEMICAL STRUCTURE OF THE COMPRTITOR HAPTEN IN THE IA DETECTABIL-ITY FOR THE IRGAROL 1051[a]

Immunogen	Group[a]	HRP-tracer[b]	Antisera IC$_{50}$s (μg/l)[c]				
			<0.1	0.1–0.3	0.3–1	1–10	>10
(structure: CONH-**KLH**; S; N N; HN N NH; cyclopropyl) **4cKLH**	I	2a		13-14-15			
		2b		13-14-15			
		2c		13	15	14	
		2d		13-15	14		
	II	2e			13-15	14	
		2f		13-15		14	
		4a			13-15	14	
		4b			13-15	14	
	III	4d	15		13-14		
		4e	15		13	14	
	IV	4c		15	13	14	

[a] Groups I, II, III and IV represent increased homology with the target analyte.
[b] See Table 7.9 for the chemical structures of the competitor haptens used as HRP tracers.
[c] The numbers 13, 14 and 15 designate the different antisera obtained against the immunogen 4cKLH. It can be observed how the chance for assays with high detectability is higher when the homology is lower.

defines IA detectability. Other authors [55,146] have in contrast suggested that different antisera-coating Ag combinations may modify the IA selectivity. In our laboratory, Oubiña et al. [55] analyzed the selectivities of three IAs for 4-nitrophenol using Abs raised against the same hapten and a battery of different haptenized CAs. The cross-reactivity studies showed a high affinity for compounds with important chemical similarities to the target analyte (as a hydroxyl and nitro group in *para* orientation), but depending on the coating Ag used, the cross-reactivity of some analytes, such as 2,4-DNP, could change from 16% to 703%. In fact, it was possible to develop an IA for 2,4-DNP using Abs raised to 4-NP that was able to recognize specifically 2,4-DNP even in the presence of 4-NP in the sample [154]. In spite of that, these authors also point on the important influence that animal immunoresponse variability may have on the properties of the resulting IA.

7.5 CONJUGATION PROCEDURES USING PROTEINS OR ENZYMES

7.5.1 Conjugation strategies

There are many proteins and enzymes to conjugate the hapten. The most frequently used as either immunogens or CAs are KLH, tyroglobulin (TG), conalbumin (CONA), BSA and ovalbumin (OVA). Similarly, HRP or AP are the most frequently used enzymes on heterogeneous IAs.

Methods used for protein conjugation have been extensively reviewed [155,156] and Table 7.10 shows a summary of some common coupling strategies. These reactions are usually made in a liquid phase, although solid-phase strategies yield excellent conjugation and easy purification procedures [157–159].

TABLE 7.9

CHEMICAL STRUCTURES OF THE ETs SCREENED DURING THE DEVELOPMENT OF IAs FOR IRGAROL 1051
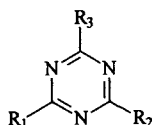
	R_1	R_2	R_3
Irgarol	NHBu^t	NHPr^c	SCH$_3$
2a	NHEt	NH(CH$_2$)$_3$COOH	Cl
2b	NHPr^i	NH(CH$_2$)$_3$COOH	Cl
2c	NHEt	NH(CH$_2$)$_5$COOH	Cl
2d	NHPr^i	NH(CH$_2$)$_5$COOH	Cl
2e	NHBu^t	NH(CH$_2$)$_3$COOH	Cl
2f	NHPr^c	NH(CH$_2$)$_3$COOH	Cl
4a	NHEt	NHPr^i	S(CH$_2$)$_2$COOH
4b	NHEt	NHEt	S(CH$_2$)$_2$COOH
4c	NHBu^t	NHPr^c	S(CH$_2$)$_2$COOH
4d	NHBu^t	NH(CH$_2$)$_3$COOH	SCH$_3$
4e	NHPr^c	NH(CH$_2$)$_3$COOH	SCH$_3$

The functional group of the spacer arm of the hapten governs the selection of the conjugation method to be used. Different functional groups are possible, but the most frequently used is the carboxylic group. The amino group has also been used in the production of Abs for mycotoxins making use of the Mannich reaction [160,161] or for the thiocarbamate herbicide molinate [12] using a diazotization method. Similarly, the alcohol group has been used to prepare the immunogen of deoxynivalenol [162]. Li et al. used a hapten halide to prepare an immunizing hapten for 4-NP [163]. The use of thiolated proteins has also been reported in the case of ceftiofur sodium [133]. Thiolated proteins are then able to react with electrophilic groups of the hapten molecule. Amino-modified dextrane has also been used as coating Ag to develop an IA for mycotoxins [164]. The amino groups react with the carboxylic groups of the hapten molecules.

It is advisable to use a different conjugation method for the immunizing hapten and the competitor. In this way, the secondary products that may be formed will not interfere in the assay reducing the background noise. Gendloff et al. developed different IAs for mycotoxins, using the same tracer but different methods of conjugation since undesired conjugation reactions using carbodiimides and the mixed anhydride method are possible [165].

Aside from the conjugation method used it is important to know the stability of the resulting bond and the yield of the coupling reaction. Thus, it has been reported lower Ab titers when an ester group was used to conjugate the hapten to the protein [166]. Similarly, imines formed when haptens possessing an aldehyde group react with the amino group of the lysine residues should be reduced with cyanoborohydride to obtain a stable secondary amine [167]. Regarding conjugation yield, it has been reported that a high ratio of haptens

TABLE 7.10

PRINCIPAL CHEMICAL STRATEGIES FOR PREPARING HAPTEN–PROTEIN CONJUGATES

Hapten R (reactive groups)	Protein P (reactive groups)	Coupling strategy	Bond type
R–COOH	P–NH$_2$ (Lysine and N-amino terminal)	1. Mixed anhydride: Isobutyl choloroformiate or isobutyl chlorocarbonate [314] 2. Active ester: NHS[a] and DCC[b], NHS and HNSA [11]	R–CO–NH–P
		3. Water-soluble carbodiimides: CMC[c] or EDC[d] [50]	R–CO–O-P (Unstable bond)
	P–OH (Tyrosine)	4. Activation using 1,2-dihydro-2-ethoxyquinoline-1-carboxylate [315] 5. Conversion to acid chloride using SOCl$_2$ [316]	
R–NH$_2$	P–NH$_2$	1. Conversion to carboxylic acid using maleic or succinic anhydride. [63]	R–CO–NH–P
		2. React with CNBr [317]	R–NH–C(=NH)–NH–P
		3. Conversion to an aldehyde. Reduction of the resulting Schiff base with NaBH$_4$ or NaCNBH$_3$ [318]	R–CH$_2$-NH–P
		4. React with thiophosgene to form the isothiocyanate [319]	R–NH–CS–NH–P
	P–Ar (Tyrosine and histidine)	1. Diazonium salt: Convert to R–N$_2$. [320]	R–NH–Ar-P
	P–COOH (Glutamic and aspartic)	1. Activation of the protein by a water-soluble carbodiimide pathway: CMC, EDC [50]	R–NH–CO–P
	P–CHO	1. Conversion the amino of protein (Lys) to aldehyde using H$_2$O$_2$ [321] 2. Oxidize the carbohydrates of glycoprotein to alhehyde with NaIO$_4$ [322] (Reduction of the resulting Schiff bases with NaBH$_4$ or NaCNBH$_3$)	R–NH–CH$_2$–P

TABLE 7.10 (*continued*)

Hapten R (reactive groups)	Protein P (reactive groups)	Coupling strategy	Bond type
R–OH	P–COOH	1. Couple using hydrazide linkage [323]	R–CO–NH–P
	P–NH$_2$	1. Convert to ester using succinic anhydride. Proceed as a R–-COOH hapten [324]	R–CO–NH–P
		2. Reaction with divinylsulfone (spacer group) [325]	R–O–(CH$_2$)$_2$-SO$_2$–(CH$_2$)$_2$–NH–P
		3. Convert to ester using chlorocarbonate or carbonyldiimidazole [325,326]	R–O-CO–NH–P (Unstable bond)
		4. React with tresyl chloride (for primary hydroxyl groups) [327]	R–CH$_2$–NH–P
		5. Coupling with epichlorohydrin (spacer group) [328]	R–CH$_2$–CHOH–CH$_2$–NH–P
R–Cl	P–SH	1. Nucleophilic attack [263]	R–S-P
R–CN	P–NH$_2$	1. Activation to imidoester with C$_2$H$_5$OH/HCl [329]	R–CNH–NH–P
R–CHO	P–NH$_2$	1. Form a Schiff base and reduction with NaBH$_4$ or NaCNBH$_3$ [167]	R–CH$_2$-NH–P
R–SH	P–SH	1. React with 2,2′-dipyridyldisulfide (thiol-disulfide interchange reaction) [330] 2. React with Na$_2$SO$_3$–Na$_2$S$_4$O$_6$ [331]	R–S-S–P

[a] *N*-hydroxysuccinimide.

[b] Dicyclohexylcarbodiimide.

[c] 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-4-toluene sulfonate.

[d] 1-(3-(Diethylamino)propyl)-3-ethylcarbodiimide.

to protein for the immunogens increases the strength and specificity of the immune response, but for competitors, a moderate value is more desirable. However, this consideration is not of general application, since excellent Abs have been obtained when few moles of hapten per mole of protein were attached. Thus, in our group high-affinity Abs for atrazine were obtained immunizing with a conjugate with few hapten molecules attached (control BSA conjugate hapten density was 4.8) [51]. With these Abs, an ELISA was developed, able to detect as low as 9 ng l^{-1} in water samples. On the other hand, an ELISA for 4-NP was developed with a detection limit of 0.61 μg l^{-1} using a coating Ag with a hapten density of 24.2, much greater than that of the immunogen (2.4 of hapten density) [55].

Verification of the coupling reaction is mainly accomplished by spectrophotometry, although this method is not useful when the hapten does not have a convenient cromophore. Moreover, measurements are often inaccurate producing an overestimation when molecules of the analyte remain non-covalently trapped into the tertiary structure of the protein. Electrophoresis offers a qualitative estimation that the conjugation has taken place whenever the hapten produces a change on the electrophoretic properties of the protein [133,168,169]. Radiolabeled haptens [11,170] have also been used, but labeled haptens are not always available and its use generates handling and waste disposal problems. Trinitrobenzenesulfonic acid (TNBS) [141] is used to evaluate free lysine residues. Studying amino acid composition [171,172] gives a quite accurate idea of the extent of the conjugation reaction. ELISA techniques [146,173] have also been used for hapten density determination but the values encountered may be underestimated if part of the covalently attached haptens are not available for Ab recognition.

During the last few years, mass-spectrometric techniques have been introduced, since they allow molecular weight estimation of high molecular weight compounds such as proteins. In this context, electrospray ionization–mass spectrometry (ESI-MS) [170,174,175] and matrix-assisted laser desorption mass spectrometry (MALDI-MS) [176] have provided reliable results. An advantage of MALDI-MS, described by Wengatz et al. [177], is that this method detects only covalently bound haptens whereas haptens bound by adsorption may also contribute to the signal in the UV-spectrum. All these methods present advantages and disadvantages, but their applicability is often limited when the carrier molecules have a high molecular size (i.e. KLH > 2000 kDa).

7.5.2 Cross-linkers

Sometimes, the total synthesis of a hapten is a tedious and costly process. The expense of a long synthetic procedure with the quality of the Abs obtained by means of an easier way must be balanced. In these cases, if the analyte structure has a suitable functional group, cross-linkers can be used. Cross-linkers are hetero- or homobifunctional molecules. Its function is to make a bridge between the analyte and the carrier, allowing its connection and avoiding steric hinderance. They can be used in many cases and only require the presence of appropriate functional groups in the analyte chemical structure (Table 7.11). Cross-linkers make the immunogen preparation easier, but they present the disadvantage of blocking important antigenic determinants in the target analyte. This strategy has been use to prepare haptens of complex molecules such as natural toxins, the antihelmintic agent hygromicin or sodium cetiofur [178–180].

7.6 IMMUNOASSAY FEATURES

In an ELISA format based on a competitive configuration (see Section 7.2), the photo-metric determination of the enzyme activity by absorption is related to the analyte concentration via a dose–response curve such as that represented in Fig. 7.5. Such calibration curves are constructed with standard analyte concentrations and have a sigmoidal shape with a linear portion. When the analyte concentration is very low, the equilibrium is in favor of a high amount of enzyme conjugate linked to the Abs, and the corresponding absorbance is maximal. The working range of the calibration curve is defined by the lower and the upper limits that can be exploited. Within this range, the change in absorbance correlates with the analyte concentration. At a higher concentration than the upper limit, the assay is saturated, and an increase in the analyte concentration no longer has an effect. Many experimental dose–response curves can be found in the literature, or are provided with commercial kits. The most common representation gives the variation of the absor-bance (B) using a logarithmic abscissa for the concentration. However, to allow direct comparison of several standard curves, the absorbance data can be normalized between 100%, which corresponds to a zero control absorbance (B_0), and 0%, which corresponds to a standard excess absorbance (B_{excess}).

Ideas on IA data processing have been well developed since at least the early 1970s and it is fair to say that all the main questions were settled by around 1975 [181–183], although there were some important contributions in the early 1980s. Various mathematical trans-formations have been proposed for a better linearization of the standard curves [184,185]. In theory, there are a very large number of choices of curves and models; in practice there are perhaps eight distinct methods which fall into three groups [184]. Some companies that sell IAs provide microprocessors that automatically convert IA optical readings to sample concentration, and the transformations are given in the commercial characteristics of the kit. One representation often selected for the microprocessors has the form logit versus log concentration

$$\text{logit}(\%B/B_0) = \ln[(\%B/B_0)/100 - (\%B/B_0)]$$

but the most used transformation is the four logistic parameters model [182] defined by the following equation:

$$y = (A - D)/[1 + (x/C)^B] + D$$

where y is the response (absorbance) and x is the dose. The values A and D correspond to the upper (maximum absorbance) and lower (theoretical non-specific binding (NSB(factor) asymptotes of the curve, respectively. The parameter B (slope factor) is related to the slope of the curve at the inflection point. Parameter C is the dose that corresponds to the center, or inflection point, of the curve.

With a dose–response curve it is possible to characterize the IA regarding detectability and sensitivity. For quality control, precision, accuracy and specificity, further experi-ments are required that will be described below. However, special attention also has to be paid to the material employed to run the assays such as microtiter plates, pipettes, batch of the biological reagents, stability, storage conditions, buffers, etc.

TABLE 7.11

LIST OF SOME OF THE MOST FREQUENTLY USED HOMO- AND HETEROBIFUNCTIONAL CROSS-LINKERS

Homobifunctional
1. Amino group

        1.1. Bis-imidoesters
        (Bisimidates)
        1.2. Bis-N-succinimidyl
        derivates
        1.3. Aryl halides
        1.4. Acylating agents

                1.4.a. Diisocyanates and
                Diisothiocyanates
                1.4.b. Sulfonyl Halides
                1.4.c. Bis-Nitrophenol Esters
                1.4.d. Acylazides

        1.5. Dialdehydes
        1.6. Diketones

2. Sulfhydryl group

        2.1. Mercurial reagents
        2.2. Disulfide forming reagents
        2.3. Bismaleimides
        2.4. Alkylating agents

                2.4.a. Bishaloacetyl derivatives
                2.4.b. Dialkyl halides
                2.4.c. *s*-Triazines
                2.4.d. Aziridines
                2.4.e. bis-Epoxides

3. Carboxyl group

        3.1. Bisdiazomethylene
        derivatives

4. Phenolate and imidazolyl group

        4.1. Bisdiazonium reagents

5. Guanidinyl group

        5.1. *p*-Phenylene diglyoxal

Heterobifunctional
1. Amino and sulfhydryl group

        1.1. *N*-succinimidyl derivatives
        1.2. *p*-Nitrophenyl esters
        derivatives
        1.3. Imidoesters
        1.4. Acyl azides and acyl
        chlorides
        1.5. Aryl and alkyl halides
        1.6. Haloketones

2. Carboxyl and ether sulfhydryl
   or amino group

        2.1. Diazoacetyl groups

3. Carbonyl and sulfhydryl group

        3.1. Alkoxylamino groups
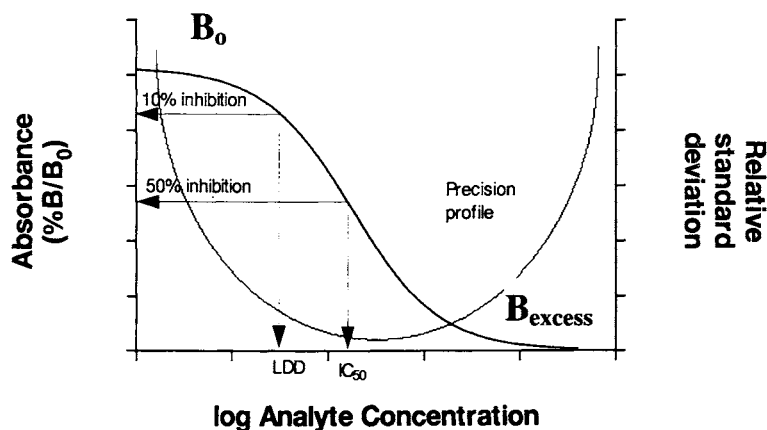
Fig. 7.5. Typical sigmoidal dose response as measured by the absorbance of the solution after incubation of a fixed concentration of antibodies and enzyme conjugates, with increasing concentration of analyte. Definition of usual parameters: limit of detection or least detectable dose (LDD), concentration inhibiting 50% the signal at zero-dose (IC_{50}). B_0 and B_{excess} are the absorbances of the zero control solution and of the standard excess solution.

7.6.1 Detectability and sensitivity

Although both terms are often confused, sensitivity is defined by the capacity to distinguish slight changes in the concentration, corresponding to the change in response (absorbance) per unit of reactant (analyte concentration). In contrast, detectability of an IA is usually expressed either as the limit of detection or as the smallest concentration of the analyte that produces a signal which can be significantly distinguished from zero for a given sample matrix with a stated degree of confidence. The minimum detectable concentration is often known as the least detectable dose (LDD) (see Fig. 7.5). Although there is no standardized way of defining the detectability, there is a general consensus in favor of selecting the dose which inhibits 10% of the binding of the Ab with the enzyme tracer at 90% B/B_0. The LDD is then often measured using the standard samples in pure water or buffer. The minimal detectable dose has also been described as two or three times the standard deviation from the mean measurement of the blank dose signal. Many scientific papers refer to the detectability of their assays as the IC_{50} value.

7.6.2 Precision

The precision of an IA is defined as the extent to which replicate analysis of a sample agrees with each other. The reproducibility is the ability to yield the same result within analysis, between analysis and between operators. According to the non-linear shape of the dose–response curve, the variance is non-uniform, and the experimental errors increase towards the two limits of the measuring range, especially in the non-linear parts (see Fig. 7.5). Therefore, the precision should be given calculating the standard deviation per percent of the coefficient of variation (CV) vs. concentration. The highest precision is

obtained for concentrations close to the concentration obtained at $B/B_0 = 50\%$. The precision profile is the best way to determine the dynamic range of an assay.

7.6.3 Accuracy

The accuracy of an assay reflects its ability to measure true values for an analyte. The dose–response curves are usually constructed with standard solutions. Another easy test consists of preparing several dilutions of a real sample and simply measuring them. Ideally, in the absence of interfering substances, the standard curve should be parallel to the curve obtained by diluting the sample within the working range. The most used method is to calculate recoveries by spiking real sample with known amounts of the target analyte. However, the best evaluation of the accuracy is obtained by comparing the results obtained from real samples with other validated analytical techniques.

7.6.4 Specificity and cross-reactivity

We mentioned before (Section 7.4.1.1) that the chemical structure of the immunizing hapten is very important for the specificity of the IAs. The specificity of an assay reflects its ability to produce a measurement of the analyte to be determined in the presence of other compounds. Cross-reactivity is defined as a specific interference related with the Ab performance and it is best described as the inability of the assay Ab to discriminate flawlessly between the analyte and a related molecule. Depending on the chemical structure of the hapten used for immunization and the class of chemicals under investigation, Ab recognition of compounds similar to the analyte is frequently observed. Therefore, it should be determined which compounds cross-react and to which degree [186]. CR of a particular Ab determines its applicability. Immunoassays with low CR values are suitable for single compound analysis. In contrast, a group-specific assay requires high CR values. Thus, the selection of the Ab depends on the purpose of the application.

Several methods to calculate CR have been described and reviewed, but the most prevalent is the 50% displacement method, originally introduced by Abraham [187]. In this method, the concentration values of standard and cross-reactant necessary to displace 50% of the bound tracer are compared. A ratio of the resulting concentrations can be referred to as %CR at the $IC_{50.}$

$$\%CR = (IC_{50} \text{ analyte}/IC_{50} \text{ crossreactant}) \times 100$$

Depending on the slope and shape of the response curve the percentage of CR (%CR) may be different at different displacement levels.

Regarding CR measurements, it is worth mentioning that in real environmental situations, a single analyte is not to be expected, but various analytes will be found together with the target compound. In this sense, the measurement of the CR should approach the real environmental situation as closely as possible. In clinical chemistry, Miller and Valdes [188] reported a model that closely approaches real situations by measuring the CR of an analyte in the presence of cross-reactants. It was proposed that one should apply various doses of cross-reactants, each in the presence of various doses of standard. This approach has been adapted to environmental CR studies of IAs for atrazine [189] and for chlorpyrifos-ethyl [190,191]. The effect of varying the concentration of the cross-reactant at a fixed standard concentration was analyzed. CR estimation, determined in the absence

of the target analyte did not correlate with the degree of interference caused by a cross-reactant in the presence of the standard. As an example, Table 7.12 shows the calculated CR of 2-benzimidazolyurea respect to carbendazim and benomyl as standards.

Knowledge of CR can be a valuable tool for analyzing a cross-reactant using an ELISA designed to monitor a different analyte, provided that this analyte is not present in the sample. In this way, a commercial ELISA for detecting parathion was used to study the disappearance of fenitrothion (a cross-reactant) under real environmental conditions in rice crop waters [192].

7.7 MATRIX EFFECT

The matrix effect could be defined as an induced deviation from theoretically predicted assay parameters, caused by constituents or properties of the sample other than the analyte in a real sample. In many cases the interfering substances are completely unknown [193]. The ultimate objective of any IA is to obtain correct results when measuring a particular analyte in a real sample; however, there are some factors, included in the matrix effect notion, which in case of being uncontrolled, can be avoided from achieving this aim. Because the IA actually is an in vitro biochemical reaction, many physicochemical conditions can influence IA performance, i.e. pH, type of buffer, reaction mixture constituents, ionic strength, temperature, etc. All these factors may have an influence, not only on the Ab–Ag interactions, but also on the enzyme performance and therefore, should be the subject of evaluation studies.

Interferences on IA methods can be categorized into two major classes:

1. Specific interferences (those substances which affect binding of Ag by competing for the specific binding sites on the Ab)
2. Non-specific interferences (those which affect the binding event between the Ab and the Ag, or the enzyme activity in a general way)

7.7.1 Specific interferences

7.7.1.1 Cross-reactivity
This referrs to those kinds of sample components which are recognized by the Ab. Usually these interferences can be predicted knowing the chemical structure of the immunizing hapten. However, specific studies have to be made which have been already discussed in Section 7.6.4.

7.7.1.2 Enzyme inhibitors

They are sample components with a direct and specific inhibitory effect on the enzyme label. For instance, in the case of HRP, primary alkyl hydroperoxides in the sample will act as substrates for the enzyme label, inducing catalysis. Since HRP is a suicidal enzyme inactivated by the catalytic process, these peroxides of the samples can inflict a variable loss of activity in the enzymatic tracer. Furthermore, it has been observed an inhibition by several anions like azide, which inhibits the peroxidase by binding to the heme group of the enzyme [194]. Therefore, azide should not be added as antimicrobial agent to buffers

TABLE 7.12

SPECIFICITY OF THE RaPID BENOMYL/CARBENDAZIM ELISA USING CARBENDAZIM AND BENOMYL AS STANDARDS AND TWO DIFFERENT CROSS-REACTANTS (BENOMYL AND 2-BENZIMIDAZOYLUREA) AT VARIOUS CONCENTRATIONS IN DISTILLED WATER; ESTUARINE WATER WAS ALSO USED FOR CHECKING THE MATRIX EFFECT

Cross-reactants		Standards					
Compound	Concentration (μg l^{-1})	Carbendazim			Benomyl		
		LDD (μg l^{-1})[a]	IC$_{50}$ (μg l^{-1})[b]	%CR[c]	LDD (μg l^{-1})	IC$_{50}$ (μg l^{-1})	%CR
Benomyl	0	0.286	4.08	100	–	–	–
	0.1	0.374	4.36	93.60	–	–	–
	1	0.380	4.64	87.88	–	–	–
	10	1.162	21.41	19.06 (n.p.)	–	–	–
	100	n.p.	n.p.	n.p.	–	–	–
2-Benzimidazuylurea	0	0.138	3.14	100	0.085	1.83	100
	0.1	0.267	3.73	84.18	0.106	2.21	83.17
	1	0.580	5.51	56.99	0.269	3.60	50.93
	10	1.266	38.80	8.09 (n.p.)	0.693	9.78	18.76 (n.p.)
	100	n.p.	n.p.	n.p.	n.p.	n.p.	n.p
Estuarine water		0.287	3.98	78.78	0.266	3.75	48.91

[a] LDD, least detectable dose calculated at 90% $B/B0$ (in μg l^{-1}).

[b] IC$_{50}$, 50% inhibition concentration (50% $B/B0$) (in μg l^{-1}).

[c] CR(%), percentage of cross-reactivity determined by estimating the amount of compound required to displace 50% of the enzyme conjugate to the amount of carbendazim. n.p., non-parallelism.

used in EIA with a peroxidase tracer. Some cations such as Ca^{2+} can lead to an activation of the peroxidase [186].

7.7.2 Non-specific interferences

These types of interference are due to other components that bear no structural resemblance to the analyte at all or physicochemical parameters, such as ionic strength, pH, salts, organic compounds, etc. An increase of ionic strength usually leads to a reduction of electrostatic interactions between Ab and Ag, because charges taking part in those interactions are shielded by the ions present [195]. The IA performance in solutions with different ionic strength should be checked, since it may change from one analyte to another depending of the predominating forces present in the immunocomplex. Thus, an IA for atrazine [51], tolerated PBS concentration between 0.1 and 1.0 M of PBS (see Fig. 7.6) while IAs for phenolic compounds were very much influenced by the ionic strength of the sample [55,142,154].

On the other hand, non-covalent forces can also be affected by pH. Some assays are very sensitive to pH effects, while others perform well in a wide range of pH. For example, assays for phenolic compounds were completely inhibited below pH 5.5. [55,163] and worked very well at basic pH. In contrast, some triazine IAs, such as the ones for atrazine and Irgarol [139,196], have been reported to work very well between pH 2.5 and 10.5 (see Fig. 7.7).

7.7.2.1 Other non-specific interferents

A troublesome class of non-specific interferents are very low molecular weight molecules such as chaotropic ions (SCN^-, I^-, Br^-, Cl^-), which may alter the 3-dimensional structure of the Ab or break the superficial interactions Ab–Ag. Other interferents are organic acids like propionic or acetic acid that have been reported to break Van der Waals interactions [195]. ELISAs for carbofuran and chlorpyrifos showed a high tolerance to the presence of a wide range of inorganic cations and anions in water and soil samples [197,198].

On the other hand, humic substances may bind non-specifically to the Ab, leading to false-positive values. Furthermore, humic acids may interact with pesticides by mechanisms involving either physical sorption or chemical reaction [199,200].

When coupling IA with extraction procedures, it is necessary to consider the organic solvents used during a previous clean-up step as potential interferents of the IA test. In an ELISA for carbaryl, the tolerance level to organic solvents was studied, showing that moderate levels of such solvents have a clear influence in the assay (see Fig. 7.8) [201]. Other authors have reported the same kind of interferences due to organic solvents [197,202]. However, the extent and direction of the solvent effect may be different from assay to assay. Thus, Sugawara et al. [203] reported the positive effects of dimethylsulfoxide (DMSO) over the detectability of an IA for dioxins.

7.7.3 Application of IAs to the analysis of environmental matrices

Since pesticides are widely employed, it is expected to find them inside a high number of matrices, mainly water, soil and food, but it is also possible to check the
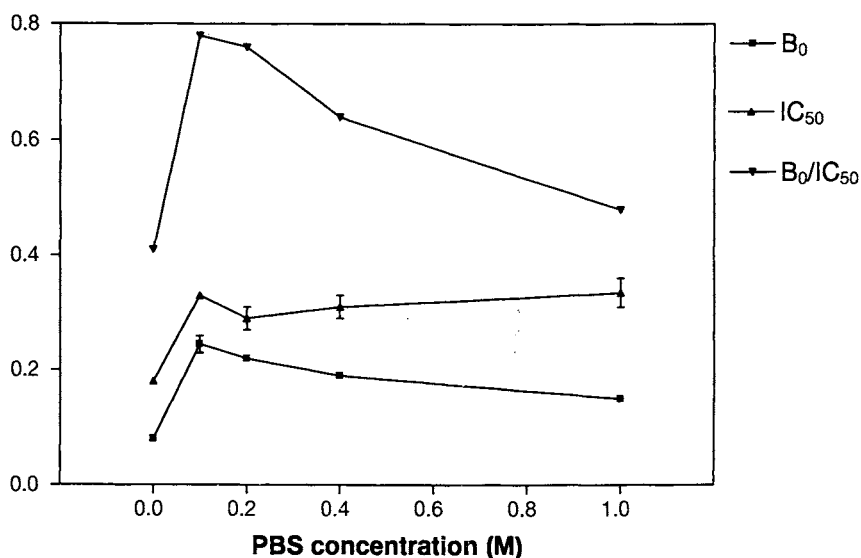
Fig. 7.6. Effect of the ionic strength on the atrtazine IA performance. From Gascón et al. [51].

presence of these compounds even in human fluids and tissues. Regarding water and soil matrices, probably the most studied types of matrix, almost all the possible interferences which have been previously referred can be found (salts, pH, cross-reactants, enzyme inhibitors, humic substances, etc.) and almost every pesticide has been tested in this matrix, e.g. atrazine [199], nitrophenol [55], organochlorine compounds such as endosulfan [141], organophosphorus pesticides such as chlorpyrifos [198] or diazinon [202], etc.
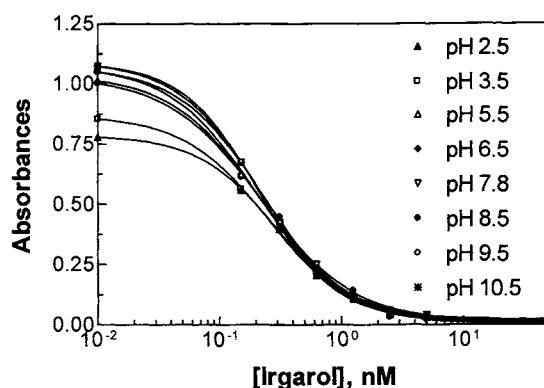
Fig. 7.7. Effect of the pH on the IA for Irgarol 1051. The ELISA is operative between pH 2.5 and 10.5. Only a small decrease of the sensitivity and of the B_0 was observed at acidic pHs whereas no significant changes occurred at basic pHs. Assays were run simultaneously on three different ELISA plates. Agreed to and accepted by ACS Copyright Office [139].

$$[IC_{50}buffer/IC_{50}solvent] \times 100$$

% solvent in buffer
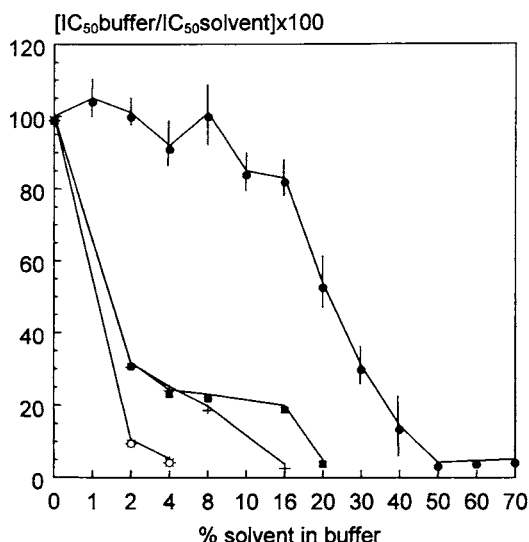
Fig. 7.8. Presence of small amounts of organic solvents in the ELISA alters the characteristics of the assay. Acetone, acetonitrile and poly(polypropylene glycol) 1000 produce a dramatic negative effect on the sensitivity of the carbaryl assay. Of the solvent tested, only methanol seemed to be tolerated when the concentration remained below 10% (v/v) in the assay buffer. Datum points represent the average of three wels. Coefficients of variation averaged are less than 2% and the standard deviations are shown for methanol. (☆) Poly(propylene glycol) 1000; (+) acetone; (■) acetonitrile; (O) methanol. Agreed to and accepted by ACS Copyright Office [201].

7.7.3.1 Water samples

IAs have been intensively used for the determination of pesticides in surface [11,204], rain [205,206] and ground water [207–210]. Mouvet et al. [211] evaluated IA operational features (cross-reactivity, detectability and reproducibility) of several IA kits for the determination of triazines in surface and ground water. Within-assay coefficients of variation (CV) were below 7%, while inter-assay CV was always lower than 20%. Results obtained with different water matrices were compared to those obtained by gas and liquid chromatography (GC and LC) to obtain the best correlation for the surface water samples. In a similar study, Thurman et al. [212] used a commercially available IA to carry out analysis of triazines in surface and ground-water samples comparing the results with those obtained by gas chromatography–mass spectrometry detection (GC–MS) after solid-phase extraction (SPE), obtaining correlation coefficients greater than 0.90.

Carbaryl and its main degradation product 1-naphthol were also determined in well-water samples from Almeria (Spain) for several months using ELISAs previously developed for these analytes [136,201,213]. The results matched very well with those obtained when applying the EPA method 531.1. In May–June the carbonyl levels of some wells exceeded the upper limit of 0.1 μg l^{-1} established by the European Community for drinking waters, corresponding to the time when applications of pesticides started in that region. Levels were again low in July, indicating either movement of the pollution

or degradation of the pesticide. Thus, 1-naphthol was detected specially after field treatments in July–August.

Seawater samples have also been analyzed by immunochemical methods. Atrazine was analyzed in estuarine and coastal waters by a magnetic particle IA (High-Sensitivity RaPID ELISA, Ohmichron Corp.) and by on-line solid phase extraction-liquid chromatography-diode array detection (SPE-LC-DAD) [196]. It was noted in this study that repeated injections of samples with a high salinity (15–35 g l^{-1}) caused broadening of the peaks in the chromatogram and therefore problems for quantifying small atrazine concentrations. This was attributed to a lifetime reduction of the precolumn because of the high salt concentration. The chromatographic method showed high precision and was more accurate since recovery values were always close to 100%. However, for high salt content samples this was only true when frequent renewal of the precolumn was performed. In contrast, several replicates of the same sample could be performed by IA since it was not affected by extreme salinity conditions. Similarly, the antifouling agent Irgarol 1051 has been determined in enclosed seawaters of the Mediterranean Spanish Coast by an ELISA recently developed in our laboratory [139,214]. A monitoring survey was performed using ELISA and on-line SPE–LC–DAD during 1996/97 with monthly sampling at the same coastal area. There was a good agreement between both techniques when measuring levels ranging from 0.007 to 0.325 μg l^{-1}.

7.7.3.2 Soil samples

Several pollutants have been analyzed in soil by immunochemical techniques (triasulfuron [215,216], triazines [85,217–219], PCBs [53,220,221], chlorpyrifos [222,223], pentachlorophenol [224], bromacil [17], etc). In this matrix, pollutants have to be extracted with organic solvents although on a few occasions water has also been used for very polar compounds. As mentioned above, the effect of the organic solvent over the IA features has to checked. Usually IAs tolerate small amounts of solvents which are miscible with water and the detectability is good enough to allow dilution of the solvent extract. Supercritical fluid extraction (SFE) is an excellent alternative to be combined with IA analysis. Wong et al. compared SFE with solvent extraction of parathion and 4-nitrophenol from soil. The extracts could be measured directly by IA without any solvent exchange.

Bound pesticide residues in soil have also been detected by immunochemical methods. Some pesticides bind organic matter of soil, mainly humic and fulvic acids and cannot be analyzed by common extraction methods. Ulrich et al. [225] developed a non-competitive sandwich IA for the analysis of bound residues based on a humic acid Ab and a triazine Ab. Humic acids were extracted from the soil, bound to the plates and the non-extractable triazine residues were detected by HRP-labeled atrazine Abs. Similarly, Dankwardt et al. [200] used a competitive IA for the investigation of bound residues.

7.7.3.3 Food samples

IAs have been applied to monitor a wide variety of environmental pollutants in food matrices. For instance, carbofuran [226], aldicarb [227], thiabendazole [228] and azinphos-methyl [229] have been analyzed in fruit juices. For example, a validation of a

carbaryl IA has been reported for the analysis of banana, carrot, green beans, orange, peach and potato extracts [230]. The analyses were carried out diluting the methanolic extracts 1:50 in PBS buffer. This dilution factor had negligible effect in the ELISA reaching sensitivities varying from 3.9 to 5.7 μg l^{-1} in these conditions. An excellent correlation was observed when compared the results with those obtained by liquid chromatography–diode array detection (LC–DAD). However, it has been demonstrated that analysis can also be directly performed in fruit and vegetable juices by just diluting them with the assay buffer and adjusting the pH of the final solution [231,232].

Aldrin and dieldrin [233], paraquat [234] and benzoylphenylurea insecticides [146] have also been analyzed in milk. A dipstick IA using MAb was used for the determination of atrazine in milk and other food samples yielding excellent recoveries [83]. The total assay time was 25 min and the dynamic range was between 0.3 and 10 μg l^{-1}.

7.7.4 Biological monitoring by IA

Monitoring of human exposure to pesticides and other environmental pollutants has been carried out by IA measuring parent chemical and/or metabolites, protein or DNA adducts. Tissues can be analyzed but usually body fluids such as blood, milk, urine, sweat or expired breath are preferred to assess individual exposure.

Most of the IAs developed have sufficient detectability for biomonitoring. Thus, an IA to analyze 1-naphthol was developed showing and IC$_{50}$ of 72 μg l^{-1} [136]. However, the urine of agricultural workers and formulators in contact with carbaryl had levels of 1-naphthol ranging from 0.07 to 1.7 mg l^{-1} and 6.2 to 78.8 mg l^{-1}, respectively. An IA for 3,4,5-trichlor-2-pyridinol, the main metabolite of chlorpyrifos in urine, was developed with an IC$_{50}$ of 0.12 μg l^{-1} [151].

The effects of the urine matrix on the detection of *p*-nitrophenol, a metabolite of parathion, by ELISA have been reported [235]. The presence of urine during the Ab–analyte interaction increased the apparent IC$_{50}$ value and inhibited color development. However, because of the high detectability of these IAs, analysis was still possible by diluting the urine with the assay buffer until a 5% concentration value was reached. When testing the matrix effect of the urine in an IA for determining metabolites of naphthalene it was found necessary to dilute the urine [135], although in this case the IC$_{50}$ was not affected. However, the authors suggested overcoming this effect by changing the ionic strength of the assay buffer.

IAs for other urinary metabolites have been reported such as the mercapturic acid conjugate of atrazine [173,236], hydroxyatrazine [48], paraquat [62], picloram [62], etc.

7.7.5 Solutions to overcome the effect of the matrix

There are several examples of successful countermeasures to overcome the matrix effect in IAs, such as sample purification, dilution, high-performance liquid chromatography (HPLC), stepwise addition and other changes in assay format. When developing a new IA or testing an analyte in a matrix with unknown effects, possible problems due to interferences should be determined in order to find the most suitable countermeasures. Classically, this analysis has been carried out by comparing a standard curve to another one in which the substance supposed to cause some interference is incorporated. If there is no matrix effect, no differences should be observed between the parameters of both curves.

Another possible method is to measure fortified samples and compare the expected and measured recovery values. These strategies are only possible if a blank sample matrix is available. In an IA with interferences, recovery suffers from deviations (it should be around 100%) and differences between observed and measured values usually decrease with dilutions.

Diluting the sample makes it possible to avoid a negative effect from the matrix. As an example, in an IA for aldicarb determination, it was not possible to eliminate the matrix effect of lemonade juice extracts unless the samples were diluted 50 times or greater [227]. In the same way, honey samples being analyzed using a flow-through immunosensor had to be diluted to 1 g l^{-1} [237]. In a MAb-based ELISA for the determination of carbaryl in apple and grapes juices, a sample dilution of 1:64 of apple juice was found to be suitable for ELISA, but it was not sufficient to analyze grape juice [231]. However, matrix dilution has a direct influence over the assay sensitivity.

Using clean-up procedures such as solid-phase or liquid–liquid extraction it is possible to remove interfering substances from samples while preconcentrating the target analyte, if desired. The procedure is then time-consuming and handling of many samples becomes more complicated. In this case, solvent compatibilities with IA should be checked.

Matrix effects have also been dealt with by adding known amounts of standard to the sample and extrapolating the result to zero-added standard (running a standard curve in the matrix of interest). After characterizing the dependence of observed signal on analyte concentration in that matrix, one can determine which analyte concentration produces the signal where no standard is added. This approach relies on an observed constancy in the relationship between relative absorbance and concentration for product extracts, which implies that standard curves run in the matrix are parallel to that run in the standard buffer. IA color development depends on the logarithm of the concentration, so adding a known amount of standard to a blank sample has much greater effect on the absorption than adding the same amount of standard to a positive sample. Because of this, the ratio of absorbances of a standard added to unaltered sample can be used to classify a sample as positive or negative [238].

7.8 VALIDATION STUDIES

The process of validating a method cannot be separated from the actual development of the method conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed. The development and validation of a new analytical method may therefore be an iterative process. Results of validation studies may indicate that a change in the procedure is necessary, which may then require revalidation. During each validation study, key method parameters are determined and then used for all subsequent validation steps [239].

Although there is general agreement about which type of studies should be done, there is great diversity in how they should be carried out [240]. The literature contains diverse approaches to performing validations [241–243]. The acceptance of IAs is dependent upon the demonstration of quality and validity compared to more traditional methods. The validation of results given by IA is usually performed by comparison with chromatographic methods, when available, and if possible with real samples. The validation guide-

lines depend on whether the IAs are used to complement the traditional analytical methods or to replace them. For quantitative methods, confirmation of the limits of quantitation, delineation of the quantitative range, evaluation of interferences and estimation of the accuracy and precision with field samples are required. When a sample pretreatment (extraction, clean-up, etc.) is required, it should be included in the method validation and described in the written procedure. Information to be included in written immunological analytical methods can be found in Mihaliak et al. [244], who describe the guidelines for using IAs in support of pesticide registration.

Immunochemical methods are particularly well adapted to environmental fate studies, i.e., of aquatic and terrestrial field dissipation, ground water, and run-off studies. Environmental surveys provide a good opportunity of comparison with chromatographic measurements in validation studies. Most of these validation studies using real samples contaminated by the analyte are devoted to atrazine, since this herbicide is found everywhere in the world and is, of course, included in most environmental surveys [34,196,204, 211,219,245–250]. A good example corresponds to a survey of 750 water samples collected from four streams in USA, with a set of 224 of them which have been also analyzed by GC–MS [249]. No false negative was observed and only 5.5% of the assays gave a false positive using ELISAs. Good correlation between both techniques was obtained in similar studies performed with alachlor [246,248,251,252], metolachlor [253] and carbofuran [197]. Marco et al. [213] also validated two IA methods for environmental monitoring of carbaryl and 1-naphthol in ground water samples with liquid chromatrography–post column reaction–fluorescence detection (LC–PCR–FD).

Validation studies for analytes less commonly detected in environmental matrix are performed with spiked samples. Oubiña et al. [223] evaluated an ELISA for the determination of chlorpyrifos-ethyl and compared the result given by IA with those obtained by automated on-line solid-phase extraction followed by LC–DAD in spiked estuarine waters, yielding a correlation of 0.991. Good correlations have been found using IA for the determination of chlorothanil as compared with the gas chromatography flame ionization detection (GC–FID) method, and for carbendazim as compared with LC determinations [254].

7.9 CONCLUSIONS AND FUTURE DEVELOPMENTS

IA has proven to be a very useful technique for the screening of contaminating substances in environmental samples. In spite of this IAs are not free from limitations (see Table 7.5) and are not yet fully accepted methods. The increasing number of target substances for which assays have been developed and the enormous number of publications of this topic will help in introducing them into the analytical laboratories. The strength of this technique lies in the possibility to screen a large number of samples (screening methods) within a short time at low costs.

Further promising developments are multi-analyte immunochemical systems wherein more than one compound or group of compounds can be detected simultaneously. Much effort should be put into the development of continuous measurements, such as FIIA and immunosensors [255]. The IA-based dipsticks are other topical and relatively simple sensing devices [83]. Also, new strategies for Ab production are being developed. As a

result of more stringent rules for the use and handling of experimental animals, conventional Abs will increasingly be replaced by Abs that can be produced with in vitro methods. Genetically engineered Ab appears to be very attractive because its selectivity and affinity can be tailored by site-directed mutations without requiring new immunizations [256]. A promising goal is the completely synthetic production of binding proteins or other synthetic receptors that are fitted to the structure of the analyte by molecular design. The use of libraries guarantees that bottleneck Ab production be closed. Also, Abs with special properties such as resistance to matrix effects or organic solvent stability could be selected from the libraries, providing an important contribution to the analysis of water and food samples. Finally, it must be brought to the readers' attention that the technical development should be accompanied by some officially organized efforts to recognize the biochemical techniques as validated, practicable routine methods. In this context, the preparation and supply of certified materials will play a decisive role in the near future.

ACKNOWLEDGEMENTS

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Chapter 8

Coupled-column LC (LC/LC) in environmental trace analysis of polar pesticides

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CONTENTS

8.1 INTRODUCTION

Multidimensional chromatography, involving orthogonal separation principles, and performed spatially in different directions, offers the highest selectivity [1,2]. However, orthogonality in coupled-column systems provides some drawbacks. For example, the difference in the physico-chemical basis of the separation processes often leads to poorly compatible mobile-phase systems, thus requiring complex interfaces.

Another drawback of applying the separation power of columns with different chromatographic separation mechanisms (multi-modal) is the occurrence of a (partially) reversed elution order of analytes and/or interferences on the two sequential columns. Hence, the separation obtained on the first column can, partly at least, be nullified on the second column.

For the reasons given, multi-modal coupled-column systems require small transfer volumes. Therefore, the application range of multi-modal coupled-column techniques will be determined by the separation power of the first column: low resolution favors

multi-residue methods (MRMs) while high resolution leads to methods for single analytes (SRMs) or a group of compounds with fairly similar properties.

In comparison with true multi-modal techniques, the coupling of chromatographic columns with similar separation mechanisms seems to be less attractive in terms of selectivity. However, the mutual compatibility of columns and the favorable compatibility to aqueous samples means, for example, that reversed-phase liquid chromatography (RPLC) employing precolumn switching (PC/LC) is a widely adopted technique for the on-line trace-analysis of polar organic micro-pollutants in water [3–9] Usually, precolumns, typically with dimensions of, 5–10 mm (L) × 2–4.6 mm (ID) are applied. In order to enable fast sampling, the precolumns are, in most cases, packed with 10–40 μm materials. The small size reduces cost, and favors a fast desorption to the analytical column.

The main purpose of precolumn switching RPLC, which is nowadays (and in this review) called solid phase extraction (SPE)-LC, is to perform a highly efficient trace-enrichment of organic micro-pollutants from aqueous samples, thus replacing laborious off-line extraction procedures. However, it does not contribute much to increasing selectivity. For the determination of some specific groups of pesticides, sorbents which have different retention mechanisms, such as ion exchange and/or, have been applied successfully, to improve selectivity in SPE-LC [10–12].

As has been clearly demonstrated for the trace analysis of polar pesticides in environmental samples such as water and soil [13–15] coupled-column reversed-phase liquid chromatography (LC/LC) employing an analytical reversed-phase column as the first column (C-1) is a good alternative for improving selectivity in hydrophobic column switching.

In this review we will discuss and demonstrate the potential of LC/LC in the field of environmental analysis. The main features of LC/LC are considered in view of the application range of the technique, and some of the basic approaches to method-development using this technique are discussed.

8.2 COUPLED-COLUMN LIQUID CHROMATOGRAPHY (LC/LC)

8.2.1 General aspects

Reversed-phase liquid chromatography (RPLC) is a suitable technique for the determination of polar analytes in water, since derivatization is usually superfluous and in the analysis of aqueous samples, the mobile-phase system is fully compatible, enabling easily automation of the analytical procedure. The wide application range, long-term stability, ease of use, low cost, and improved selectivity (with diode-array detection) means that UV detection is widely used in residue analysis. However, in environmental analysis RPLC-UV is usually complicated by a large excess of polar interferences, e.g. anions and humic- and fulvic acids, making the determination of more polar analytes, eluting in the first part of the chromatogram, difficult or impossible. This is also shown by the simulated chromatogram in Fig. 8.1, presenting the interferences usually encountered in pesticides-residue analysis employing RPLC-UV. The most common problem is caused by abundant, early-eluting sample constituents indicated as S-1. In many cases, S-1 can be removed largely via a low-resolution clean-up such as off-line SPE or on-line with PC/LC. However, for analytes with nearby polarities in the range of the large excess of interfer-
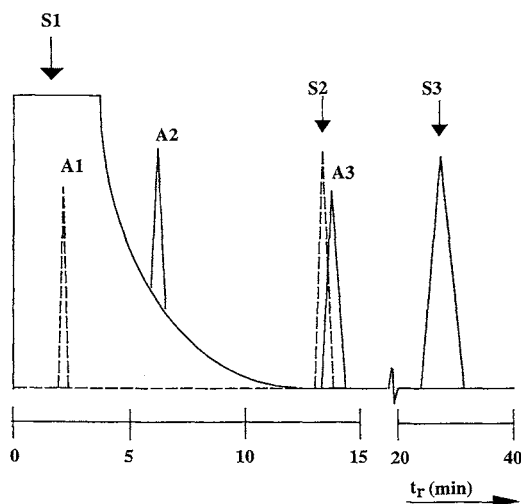
Fig. 8.1. Simulated chromatogram of sample-interferences occurring in the RPLC-UV trace analyses of organic micro-pollutants in environmental samples: S1, S2 and S3 are sample-interferences: A1, A2 and A3 are analytes.

ences, such a clean-up will not be sufficient. Compounds with longer retention times than the analytes, S-3, do not really interfere, but cause an unfavorable increase in the time of analysis. Tuning of the selectivity by small changes in mobile-phase compositions, e.g. eluotropic strength or type of modifier, is usually applied to improve the separation caused by interferences of the type S-2.

Reversed-phase coupled-column RPLC-UV (LC/LC) can effectively remove interferences of types S-1 and S-3. This is illustrated in Fig. 8.2, which shows schematically the events in an on-line clean-up performed with the LC/LC heart-cutting technique. After the injection of an aliquot of uncleaned sample or extract (event I) on the first column, C-1, a clean-up (event II) is performed with a certain volume of M-1 (clean-up volume), the mobile phase of C-1. During this event (II) a large amount of the type S-1 interferences are removed from C-1. Just before the elution of the (first) analyte takes place, C-1 is switched on-line with the second separation column, C-2, during a time when the fraction, A, containing the analyte(s) (the transfer volume) is completely transferred from C-1 to C-2. Finally, the analytes (A1, A2, A3,...) are separated on C-2 and simultaneously interferences of type S-3 will be washed from C-1 with M-1 or a stronger eluent, and reconditioned with a volume of M-1 prior to the next injection. An overview of the important parameters involved in the application of PC/LC and LC/LC in the on-line trace analysis of polar pesticides in water samples is made in Table 8.1. By comparing the parameters, several differences between SPE-LC and LC/LC can be noted.

The high sample-throughput of LC/LC originates from the fact that the sample injection is performed in the high-pressure mode, with an autosampler equipped with a large volume loop and a syringe pump. The insertion by the injector of an air gap at the front and end of the sample provides fast and accurate large volume injection with a minimal
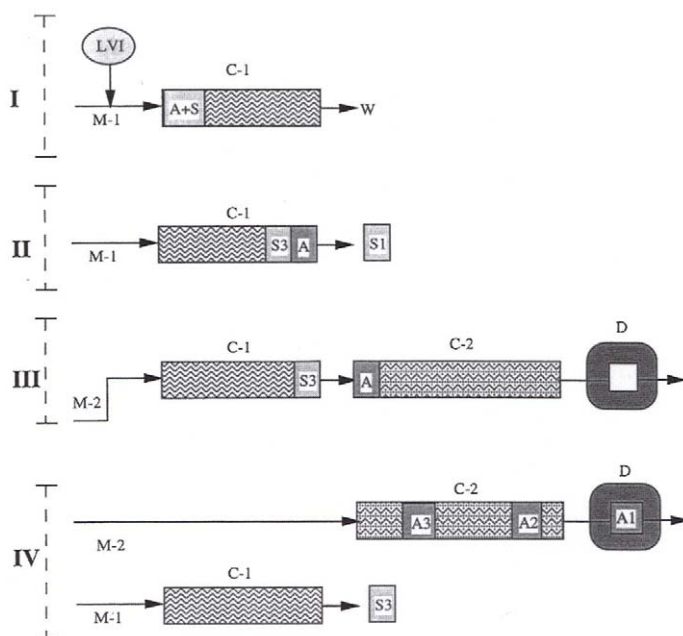
Fig. 8.2. Principal events in LC/LC. LVI, large volume injection; C-1, first separation column; M-1, mobile- phase of C-1; C-2, second separation column; M-2, mobile-phase of C-2; D, detector; S, sample interferences; A, analytes. For S1, S3, A1, A2 and A3, see Fig. 8.1.

consumption of sample. This feature allows the use of rather small autosampler vials, which facilitates the processing of large series of samples (see Table 8.1).

Because of the inherent high pressure of a full-size separation column the speed of sample loading is restricted. Hence, from both a practical point of view (design of auto-

TABLE 8.1

SYSTEM PARAMETERS OF HYDROPHOBIC SPE-LC AND LC/LC AS APPLIED TO THE ON-LINE DETERMINATION OF POLAR PESTICIDES IN WATER SAMPLES EMPLOYING LARGE VOLUME INJECTION

Parameter	SPE-LC	LC/LC
Dimension C-1		
Length (mm)	2–10	30–150
ID (mm)	2–4.6	3–4.6
Particle size in C-1 (μm)	10–40	3 or 5
Resolution C-1	Low	High
Range sample injection volume (ml)	50–250	0.2–8
Mode of sample delivery (injection)	LC pump and low pressure valve(s)	Loop filling and high pressure valve of autosampler
Vial capacity system	12–18	70–120

sampler) and the time of sampling, the sample injection volume in LC/LC is limited to about 10 ml. Consequently, the UV sensitivity of the analytes should be sufficient, typically $\varepsilon_0 = 15\ 000$ l/mol per cm, in order to obtain LODs below the 0.1 μg/l level. This means that in case of very well retained analytes the ability of SPE-LC to sample distinctly larger volumes can offer higher sensitivity than on-line LC/LC.

It should be emphasized that in our approach the forward flush mode is advocated in column switching. Although sometimes the application of backflush is described [16], this mode has two drawbacks for polar-to-moderately polar analytes. For the most polar compounds it will lead to additional band broadening, while for more-retained analytes, reversing the flow will diminish the separation obtained earlier in the process.

As shown in Fig. 8.2, the major advantage of LC/LC is the high resolution of C-1, which contributes largely to the enhancement of the selectivity by removing a large part of the early-eluting interferences. Another crucial feature of LC/LC is the transfer volume, i.e. the time that C-1 is coupled on-line to C-2. In complex samples it is unavoidable that part of the interferences will be transferred together with the analytes. Hence, the attainable selectivity will be determined by: (i), the effectiveness of clean-up on C-1 (clean-up volume); and, (ii), the volume of the analyte fraction (transfer volume, A) which limits the transfer of S3. In other words, optimal selectivity will be obtained at a minimal transfer volume, while at least part of the required separation should have taken place before transfer. The key to success in enhancing the selectivity in the analysis of polar analytes in environmental samples is the effective removal of early-eluting interferences. Therefore, the development of multi-residue methods involving larger transfer volumes, which would lead to less selectivity, can still be quite attractive.

The possibilities, features and limitations of LC/LC in environmental analysis will discussed in the next sections, starting with LC/LC-UV method-development for successful application to the on-line trace analysis of pesticides. Next the value of off-line extraction procedures in combination with LC/LC in both single- and multi-residue analysis will be demonstrated for water and soil samples. This will include the beneficial use of analytical Restricted Access Medium (RAM) columns for the improved separation between acidic analytes and the excess of humic acid interferences, which are always abundant in environmental samples. In addition, optimization procedures for the selection of optimal conditions for clean-up volume, transfer volume, the separation of analytes and chromatographic run times employing coupled-column LC with step-gradient elution are discussed. Finally, applications will be given of coupled-column LC in combination with selective detection.

8.3 LC/LC-UV

8.3.1 Practical method-development

The route usually followed in LC/LC-UV method-development is given in Fig. 8.3. The applicable UV absorption (λ and ε) and the retention of the analyte from pure water onto the C18-type stationary phase largely determine the sensitivity and selectivity that can be obtained. In our experience (see below, also), with insufficient UV-vis absorption or selectivity (ε values of less than ca. 1000 l/mol per cm and $\lambda < 200$ nm) and/or an essential absence of retention on a C18-bonded phase (k values of less than ca. 1), the
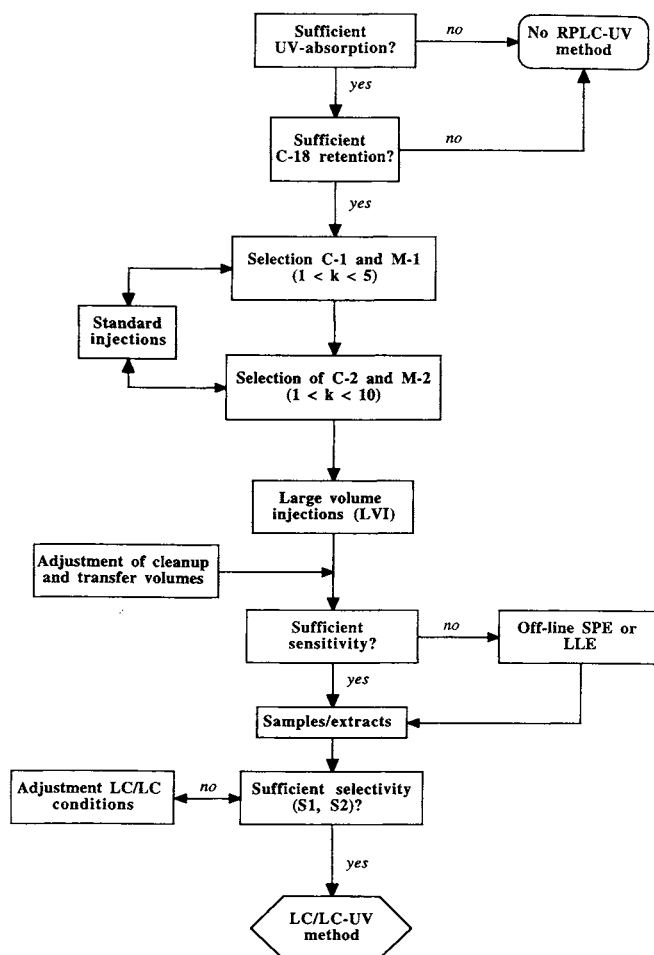
Fig. 8.3. Scheme of LC/LC-UV method-development.

successful development of an on-line coupled-column RPLC-UV procedure for trace-
level analysis becomes highly unlikely.

In our laboratory, the required information is usually obtained by a scouting experiment
which includes the RPLC-UV analysis of a small volume of a standard solution. Under
isocratic elution conditions providing $1 < k < 10$ and photo-diode array detection, the
UV-sensitivity (ε) at a suitable wavelength can be calculated easily from the analyte-peak
data in the chromatogram.

An overview of the acquired RPLC-UV information on pesticides is given in Table 8.2.
In this table the UV-absorption maximum was selected as a good compromise between
selectivity and sensitivity, as encountered in this field of analysis. Besides the UV-sensi-
tivity (ε), the peak elution volume of the analyte contributes inversely to the degree of
analyte detectability. Therefore, information on the elution of the analyte, expressed as the
column efficiency (N), is included in Table 8.2. The large variation of the elution effi-

TABLE 8.2

RPLC-UV INFORMATION ON PESTICIDES ON A 50 × 4.6 MM ID PACKED WITH 3 μm C18[a]

Pesticide	Modifier (%)	k	N[b]	λ (nm)	ε_0 (l/mol cm)
Acephate	10[c]	2.0	1100	205	1000
Alachlor	50	5.9	1180	220	9000
Alloxydim-sodium	50	8.4	3400	260	8300
Alloxydim-sodium				300	4750
Amitraz	35	2.1	900	235	3000
Asulam	25	1.5	1000	269	15500
Atrazine	35	3.2	2400	221	29000
Azinphos-ethyl	50	6.2	5100	222	25700
Azinphos-ethyl				286	7000
Azinphos-methyl	50	3.0	2900	221	24500
Azinphos-methyl				285	6150
Barban	45[c]	9.8	4900	238	17000
Benazolin	35	2.5	2300	217	35000
Bentazone	35	2.6	2350	217	25000
Benzoximate	65	5.9	4350	225	24500
Bromacil	35	1.5	950	277	7000
Bromoxynil	40	4.8	2500	220	30000
Carbetamide	45[c]	1.7	3000	234	17000
Chlorbufan	45[c]	7.3	5100	237	19000
Chlordimeform	35	2.7	1900	239	2000
Chloridazon	20	3.4	2500	229	27000
Chloridazon				285	8500
Chloroallyl alcohol	0	7.0	1100	205	10000
Chlorprofham	45[c]	9.3	5100	238	17000
Chlorsulfuron	35	6.7	5100	224	25800
Cinosulfuron	35	3.2	4600	221	24500
Cyanazin	35	1.8	1250	220	28500
Cypermethrin	80	4.0	280	230	30000
2,4-D	50	1.6	700	228	8050
2,4-DB	50	2.8	1450	229	7150
Deltamethrin	80	4.5	700	230	23300
Desmedipham	45[c]	4.8	4700	233	35000
Dicamba	20	3.0	1350	230	7300
Dichlobenil	50	5.0	4450	230	7300
2,6-Dichlobenzamide	25	2.1	1500	220	7250
Dichlorprop (2,4-DP)	35	7.1	2750	228	8500
Diflubenzuron	48	7.2	4500	260	16000
Diethofencarb	45[c]	6.6	4500	244	18000
Dinoseb	50	8.0	3630	271	11600
Dinoterb	50	9.1	4000	267	11750
Diuron	35	5.4	1260	249	21500
DNOC	35	7.2	2200	266	12500
Ethofumesate	55	3.2	5000	225	6000
Ethylenethiourea	0	1.6	1000	233	18000

TABLE 8.2 (continued)

Pesticide	Modifier (%)	k	N^b	λ (nm)	ε_0 (l/mol cm)
Fenfuram	40	4.1	2100	258	19000
Fenpropathrin	80	3.7	680	230	11350
Fenpropimorf	55^d	13.5	2850	205	20000
Ioxynil	40	6.4	2500	233	35000
Iprodion	50	5.1	3750	220	21300
Isoproturon	35	5.0	3250	240	23700
Linuron	50	4.9	2350	248	17500
MCPA	50	1.8	600	227	8000
MCPP (mecoprop)	50	2.9	1400	228	7800
Metalaxyl	50	1.6	1250	220	9900
Metamitron	35	0.9	565	308	10800
Methabenzthiazuron	35	3.6	2880	217	26000
Methabenzthiazuron				267	13450
Methamidophos	10^c	1.3	1200	205	420
Methylisothiocyanate	40	2.8	2200	237	3000
Metobromuron	50	2.0	1550	250	20700
Metoxuron	30	5.5	2500	240	16000
Metribuzin			2350	294	9260
Metsulfuron-methyl	35	4.9	5000	226	25500
Monuron	35	2.4	1800	245	16400
2-Naphtoxyaceticacid	35	3.5	1850	224	44000
1-Naphtylacetamide	35	1.6	760	220	46000
Pencycuron	55^d	7.0	3450	240	20000
Pentachlorophenol (PCP)	60	4.0	2550	220	22000
Permethrin-I	80	5.3	1000	230	21700
Permethrin-II	80	6.3	1340	230	21000
Phenmedipham	45^c	5.5	3300	237	39000
Prochloraz	65	5.9	350	220	12650
Prometryn	50	7.4	300	225	23000
Propham	45^c	3.6	5000	231	19500
Quintozene	65	8.6	800	230	19350
Sulfometuron-methyl	35	5.6	5100	235	20600
2,4,5-T	50	2.8	1100	229	8700
2,4,5-TP (fenoprop)	40	4.5	5000	230	7800
Thifensulfuron-methyl	35	4.4	4500	225	17500
Trichlopyr	50	2.3	1200	232	8500
Vamidothion	10^c	15.0	2000	205	5300

[a] Mobile phase of acetonitrile (modifier) – 0.03 M phosphate buffer, pH 3.0, at a flow of 1 ml/min.

[b] Number of plates on the column measured as $(t_r/\sigma)^2$.

[c] Methanol in water.

[d] Acetonitrile in 0.2% ammonia water.

ciency of the analytes, ranging from 280 (cypermethrin) to 5100 (chlorbufan), clearly emphasizes the importance of this parameter to sensitivity.

As indicated in Fig. 8.3, the next steps involve (if necessary) adjustment of the chromatographic conditions on the first column (C-1). This column must provide sufficient separation to remove a major part of the abundantly available early eluting matrix constituents prior to elution of the analyte. Therefore, a column with high separation power is attractive. However, an unnecessary increase of separation power and dimensions of the column must be avoided: the peak elution volume, which is inversely proportional to the sensitivity in detection of the analyte, should be kept as low as possible. An increase in the column dimensions will also increase the reconditioning time (if necessary) of C-1 between analyses. In general, an eluotropic strength of M-1 should be selected such that the capacity factor of the analyte is in the range of $1 < k < 5$, rendering a clean-up volume of at least twice the dead-volume of the column (V_o) to remove a large part of the excess of early eluting interferences.

It is favorable to select a second column (C-2) with a higher separation power than C-1, especially in the analysis of compounds with little C18 retention. This will provide some flexibility when optimising the eluotropic strength of M-2 in order to reach an adequate compromise between the required separation efficiency on the second column, and the desired peak compression (sensitivity of detection). In general, one should not 'overkill' the separation power, and in order to achieve a short time of analysis and good sensitivity the capacity factor of the analyte(s) in both mobile phases, M-1 and M-2, should not exceed 10. It must be emphasized here, that the eluotropic strength, φ (fraction of organic of modifier), of M-1 must never exceed that of M-2 ($\varphi_{M-1} \leq \varphi_{M-2}$). During this part of the optimization one can, if required, tune the selectivity by taking another type of hydrophobic column packing material or one from a different manufacturer or, first in most cases, by changing the mobile-phase constituents such as the type of modifier, the buffer and pH. The latter parameter can play a crucial role for the determination of acidic compounds and, based on experience, two additional boundary conditions seem to be important. First, in order to process large sample volumes, the initial pH of the mobile-phase should be as low as possible – for stationary phases based on modified silicas, typically around pH = 2.3. Secondly, no modifier gradient should be used, since this will result in large interference peaks (humps) caused by a constant release of humic and fulvic acids from the column during the gradient. It has been demonstrated that the use of pH-based gradients offers higher selectivity [13].

After the selection of adequate conditions the attainable sensitivity is determined by large-volume injection (LVI) of standard solutions in HPLC-grade water. This is usually done by connecting C-1 to the UV-detector, which also establishes the clean-up and transfer volume. The maximum allowable sample injection volume depends largely on the C18 retention of the analyte. For example, the small C18 retention of polar compounds such as chloroallyl alcohol (CAAL) and ethylenethiourea (ETU) (Table 8.2) makes focusing of the analyte on the top of the column impossible and, because of migration of the analyte during injection, band-broadening rapidly starts to increase with increasing injection volume. In general, the start of peak-deformation of the analyte upon injection on C-1 is selected as a criterion for the maximum allowable injection volume.

If sufficient sensitivity cannot be obtained with an LVI below 10 ml, on-line LC/LC is

not possible, and an off-line concentration step will be necessary. This approach will be discussed later.

Finally, the total procedure is used to analyse real-life water samples. Of course, depending on the type of water sample and the site of sampling, the concentration and nature of the ionic and/or highly polar interferences can vary strongly. We have indeed encountered such problems in the determination of ETU in various types of groundwater [15]. In our experience, increasing the separation power of column C-1 is most efficient.

8.3.2 Applications of on-line LC/LC-UV to water samples

An overview of the work mentioned above [13–15] and other applications [17–22] involving LC/LC-UV for the on-line trace analysis of 16 pesticides and related compounds in water samples is given in Table 8.3. The simplicity and effectiveness of the coupled-column RPLC-UV analyser provides selective methods with a high sample throughput (run times of about 10 min). As discussed above, and illustrated by the data given in Tables 8.2 and 8.3, the sample volume injection (LVI) for very polar analytes is limited. Hence, for compounds such as chloroallyl alcohol, ethylenethiuronium and vamidothion, the LVI is limited to 0.2–0.5 ml, yielding LODs in the range of 1 μg/l. By the use of relatively simple manual concentration steps the LODs can be lowered to the EC drinking-water-limit of 0.1 μg/l [17–19]. Despite the marginal separation of these very polar analytes, and the excess of early eluting interferences, the large improvement of selectivity when applying LC/LC is clearly illustrated in Fig. 8.4. Obviously, in comparison to the use of two coupled C18 columns without column-switching the LC/LC approach provides a significant reduction of the detection limit, a phenomenon also encountered in ion chromatography [23]. In RPLC, some multi-dimensionality caused by a size-exclusion effect, can be observed, since different migration speeds of the compounds on the two columns, caused by the ionic strength of the sample, plays a role [19]. For compounds with sufficient C18-retention and UV-detection (see Table 8.2) LODs of 0.1 μg/l can be obtained, employing sample injections below 8 ml (see Tables 8.2 and 8.3). Examples are given in Fig. 8.5, showing the performance of an LC/LC-UV analyser for the determination of isoproturon and methabenzthiouzuron in surface water in less than 8 min. The screening method developed for the simultaneous determination of bromacil and diuron (active ingredients of the herbicide Krovar, Dupont) for the control of a water location near citrus orchards in Mediterranean areas is a good example of improved methodology [20]. Obviously large-volume injection LC/LC-UV (see ref. [20] and Table 8.3) is distinctly more efficient than the method used before, involving a liquid–liquid extraction, Kuderna–Danish evaporation and RPLC column-switching [24]. This study reported a rather unexpected matrix-dependent retention time of bromacil on C-1. Investigations of LC-grade drinking- and surface-water, showed that differences of about 1 min were observed, indicating that one must consider this effect in LC/LC method-development.

The SRMs shown in Table 8.3 make use of small transfer volumes (typically 0.2–0.7 ml) in order to enhance selectivity. Although expected, the developed MRMs [21,22], employing larger transfer volumes for the transfer of analytes with a wide polarity range, did not suffer sufficient selectivity. This underlines the fact that in LC/LC the performance of an efficient clean-up on C-1, namely in the removal of S1, is a dominant selectivity

TABLE 8.3

COUPLED-COLUMN RPLC_UV CONDITIONS AND PERFORMANCE FOR THE ON-LINE ANALYSIS OF PESTICIDES IN WATER

Analyte	C-1[a]	C-2[a]	M-1	M-2	Injection volume (ml)	Cleanup volume (ml)	Transfer volume (ml)	UV (nm)	LOD (µg/l)	Time of analysis (min)	Ref.
Bentazone	A	B	MeOH–buffer[b], pH 2.3, (50:50)	MeOH–buffer[b], pH 2.7, (50:50)	2.0	4.65	0.45	220	0.1	8	[13]
Isoproturon	A	B	ACN–water (48:52)	ACN–water (48:52)	4.0	5.85	0.40	240	0.1	10	[13]
Linuron	A	B	ACN–water (50:50)	ACN–water (50:50)	4.0	6.6	0.4	249	0.1	12	[13]
Methabenzthiazuron	A	B	ACN–water (42:58)	ACN–water (42:58)	4.0	5.90	0.45	267	0.1	8	[14]
Ethylenethiourea	C	C	ACN–water[c] (1:99)	ACN–water[c] (1:99)	0.20	2.60	0.44	233	1.0	5	[17]
Chloroallyl alcohol	A	B	Water	MeOH–water (5:95)	0.20	1.20	0.80	205	1.0	7	[18]
Methylisothiocyanate	A	B	ACN–water (40:60)	ACN–water (50:50)	0.77	1.90	0.40	237	1	7	[19]
Vamidothion	A	B	ACN–water (15:85)	ACN–water (22:78)	0.50	3.30	0.70	205	2	8	np[d]
Metamitron	C	C	ACN–water[c] (25:75)	ACN–water[c] (30:70)	6.0	10.4	0.15	310	0.1	15	[15]
Bromacil, diuron	D	B	MeOH–water (30:70)	MeOH–water (65:35)	2.0	5.00	0.90	277	0.1	10	[20]
Simazine, atrazine and terbutryn	D	B	ACN–water (40:60)	ACN–water (70:30)	2.0	2.90	0.70	223	0.1	7	[21]
Atrazine, deisopropyl atrazine, hydroxy- and deethyl-atrazine	D	E	ACN–water (20:80)	ACN–water (35:65)	2.0	2.60	4.20	220	0.3	12	[22]
Cinasulfuron, triasulfuron, metasulfuron-methyl	A	A	ACN–buffer[b], pH 3.0 (45:55)	ACN–buffer[b], pH 3.0 (45:55)	4.0	6.50	1.70	226	0.2	12	np[d]

[a] Type of columns: A, 50 × 4.6 mm ID packed with 3 µm Microspher C18; B, 100 × 4.6 mm ID packed with 3 µm Microsphere C18; C, 150 × 4.6 mm ID packed with 5 µm Hypersil ODS; D, 30 × 4.6 mm ID packed with 5 µm Spherisorb ODS-2; E, 125 × 4.6 mm ID packed with 5 µSpherisorb ODS-2.

[b] 0.1% Phosphoric acid in water.

[c] Containing 0.2% ammonia.

[d] np, not published.
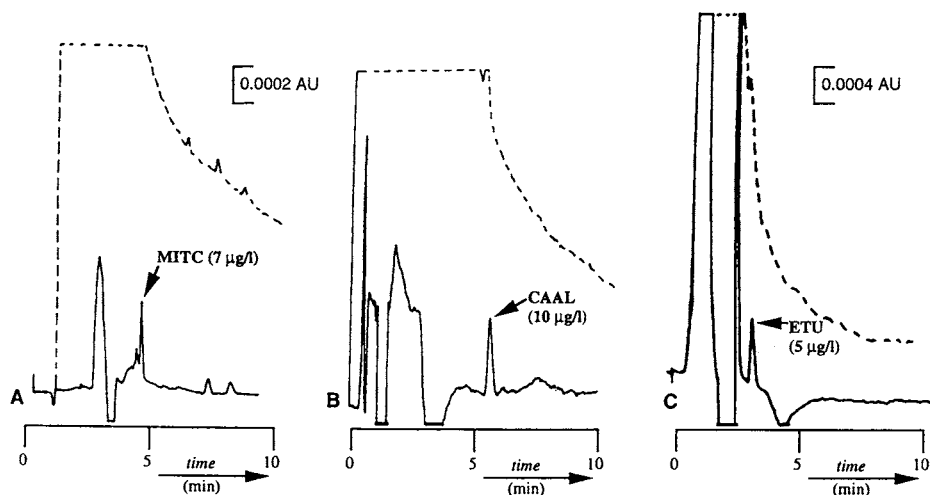
Fig. 8.4. LC/LC-UV with direct sample injection of environmental aqueous samples spiked with highly polar analytes. For LC/LC-UV conditions, see Table 8.3. Dashed lines: chromatograms obtained using two columns in series, without column switching, and using mobile-phase M-2.
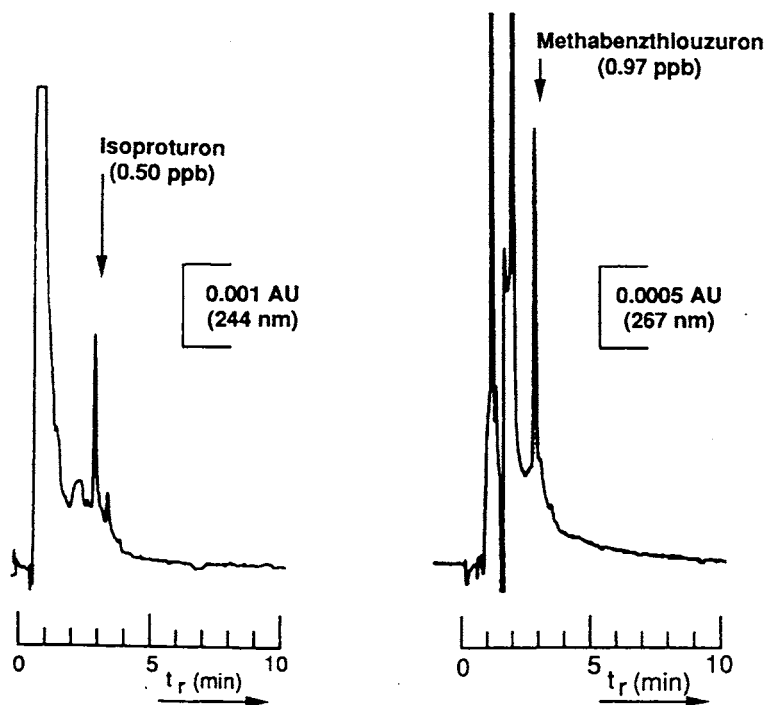
Fig. 8.5. LC/LC-UV with direct sample injection of surface water samples spiked with polar pesticides. For LC/LC conditions, see Table 8.3.
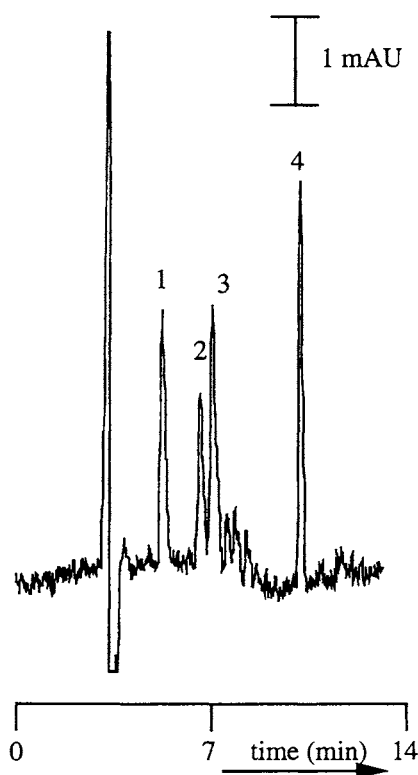
Fig. 8.6. LC/LC-UV (220 nm) of a surface-water spiked at the 2 μg/l level with (1), deisopropyla-trazine; (2), hydroxyatrazine; (3), deethylatrazine; and (4), atrazine. For LC/LC conditions, see Table 8.3.

parameter for this type of analysis. The performance of on-line trace analysis of triazines in surface water at a level of 0.4 μg/l is given in Fig. 8.6.

By comparing the on-line LC/LC-UV MRM procedures for triazines and metabolites with other reported methods, it is relevant to emphasize that small sample volumes (<2 ml) are used to achieve LODs similar to those given by on-line or off-line SPE techniques with large amounts of sample (up to 2000 ml) [25–27]. The examples discussed above show that on-line LC/LC-UV can offer robust and fast SRMs and MRMs for the direct analysis of a wide variety of compounds in environmental water samples. For analytes with sufficient UV absorption and selectivity (ε- and λ-values of more than 15 000 l/mol cm and 220 nm, respectively) and C18 retention ($k > 50$, in pure water), LODs in the range of 0.1 μg/l are feasible.

Beside the favorable features discussed above, LC/LC has some limitations. Because of its maximal sample injection volume of about 8 ml the on-line LC/LC-UV approach is not viable for pesticides with sufficient C18 retention but having ε-values below about 14 000, e.g. chlorophenoxy acid herbicides (see Table 8.2). Another drawback is that in RPLC-UV the chromatographic analysis of acidic pesticides in environmental samples is usually hampered by a broad hump originating from humic and fulvic substances. In case of
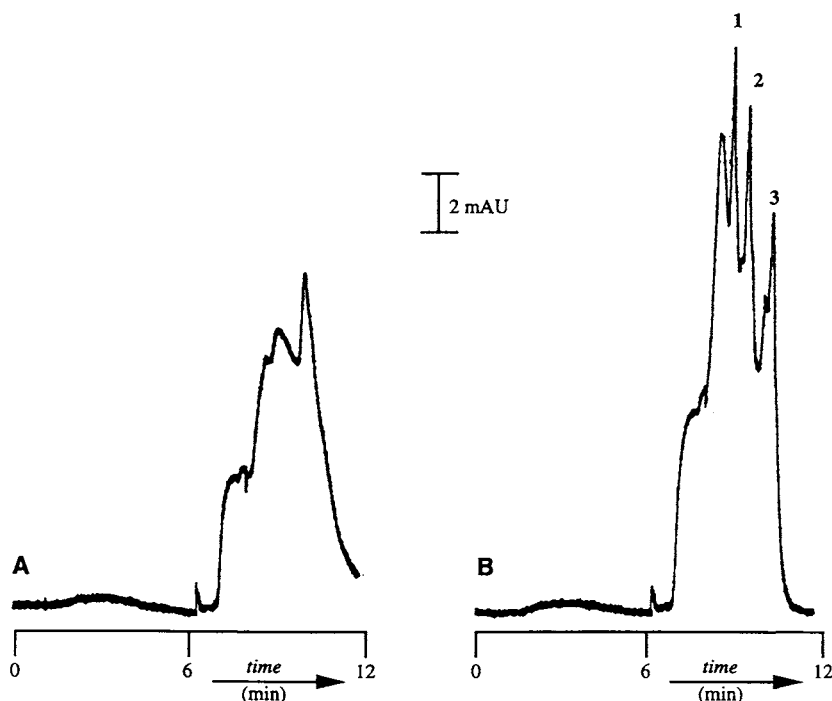
Fig. 8.7. LC/LV-UV (226 nm) of a (A) blank drinking-water and, (B), drinking-water spiked at 1 μg/ l with: (1), cinasulfuron; (2), triasulfuron and, (3), metsulfuron-methyl. For LC/LC conditions, see Table 8.3.

the LVI-LC/LC-UV analysis of bentazone the combination of a 2 ml injection volume, a small transfer volume (0.40 ml), and pH-base gradient provided sufficient selectivity (see Table 8.3). However, when increasing the sample load and/or using larger transfer volumes, the baseline deviation of the hump in the chromatogram severely hampers the quantification of acidic compounds. This effect is demonstrated in Fig. 8.7, showing an on-line analysis of a drinking water sample spiked with three sulfonylurea herbicides at a level of 1 μg/l, using LVI (3.9 ml) under optimized LC/LC-UV conditions. As can be seen, the humic hump makes it difficult to reach the 0.1 μg/l level for this rather clean matrix. In method-development, the application of pH- or modifier-based gradient did not improve selectivity. Moreover, the use of surface water, groundwater, and/or the use of larger transfer volumes for the simultaneous determination of compounds with a wider range in polarity significantly increased the deterioration of the chromatographic analysis.

8.3.3 Applications of off-line SPE and LC/LC-UV to water samples

In order to employ the favorable LC/LC-UV features (efficient removal of S1) an off-line preconcentration step will be necessary for the determination of compounds with insufficient UV-sensitivity, if detection limits below 0.1 μg/l are required. Off-line SPE procedures can offer advantages over on-line procedures in this field of analysis. For

example, organic extracts (concentrated) obtained by off-line procedures, e.g. liquid–liquid extraction or SPE, may be stored over a longer period of time so that they can be analysed as batches. This is more efficient for trend-studies involving small series of samples with a low frequency of sample collection over a long period of time. For studies involving the determination of pesticides of different chemical families, operational MRMs – as applied in monitoring programmes – are often applied in combination with GC-based multiresidue methods. It is then often more convenient to use a unified extraction–concentration procedure with an organic solvent or a (semi)-automated solid-phase extraction, so that the same extract can be used for all MRMs to be applied on a sample. Moreover, the availability of extracts easily allows one to preserve part of the sample for confirmation analysis employing derivatization and/or gas chromatographic techniques [15].

Even for compounds with favorable RPLC-UV properties an off-line SPE step can be considered in order to improve quantification at low levels. An example is given in Fig. 8.8, showing the determination of bentazone in a surface water sample containing 0.40 μg/l of bentazone. The comparison between the on-line LC/LC-UV method and the procedure including an off-line SPE step shows clearly that such a step considerably improves the sensitivity. The SPE procedure consisted of the following steps (i), the sampling of 200 ml of water (adjusted to pH of 2.2 with phosphoric acid) on a preconditioned (2 ml of methanol, 2 ml of acetone, 2 ml of methanol and 2 ml of 0.1% trifluoroacetic acid in water, (pH 2.2)) 500 mg C18 cartridge, followed by air-drying of the cartridge for about 30
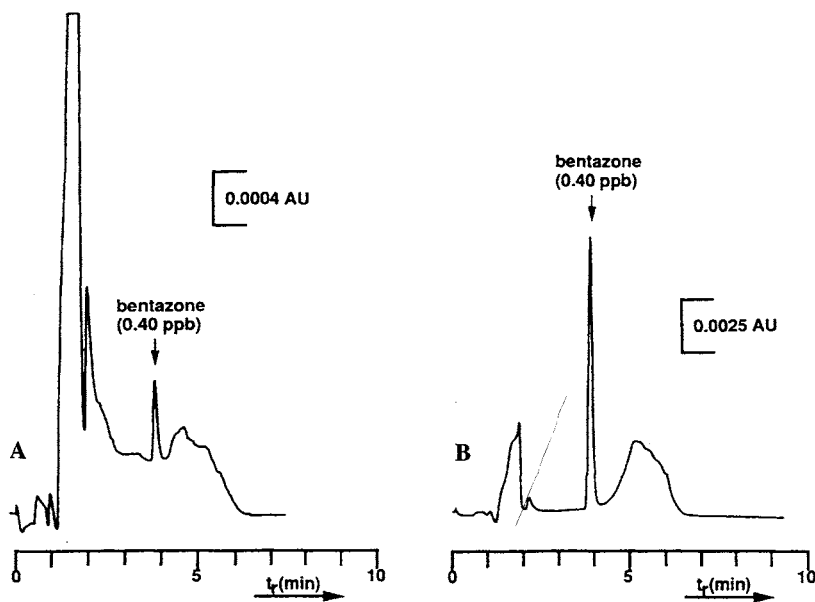
Fig. 8.8. On-line LC/LC-UV (220 nm) vs. off-line-SPE and LC/LC for the analysis of a surface-water containing 0.40 μg/l of bentazone. A, on-line LC/LC employing 2.0 ml of sample injection (for further conditions, see Table 8.3); B, injection of 200 μl of redissolved extract obtained after SPE (for procedure, see Text) and corresponding to 20 ml of water; clean-up volume, 2.45 ml of M-1: further conditions, as Fig. 8.8A.

min; (ii), the desorption of the analyte with 2 ml of acetone, and evaporation of the solvent with a gentle stream of nitrogen; (iii), the re-dissolution of the residue by adding (first) 400 μl of methanol and (secondly) 1600 μl of 0.05% TFA in water.

Improved sensitivity was required in a project involving the monitoring of bentazone in rain water down to a level of 0.05 μg/l. The RPLC-UV analysis of a rain water sample containing 0.05 μg/l of bentazone (Fig. 8.9) demonstrates that quantification at this level is
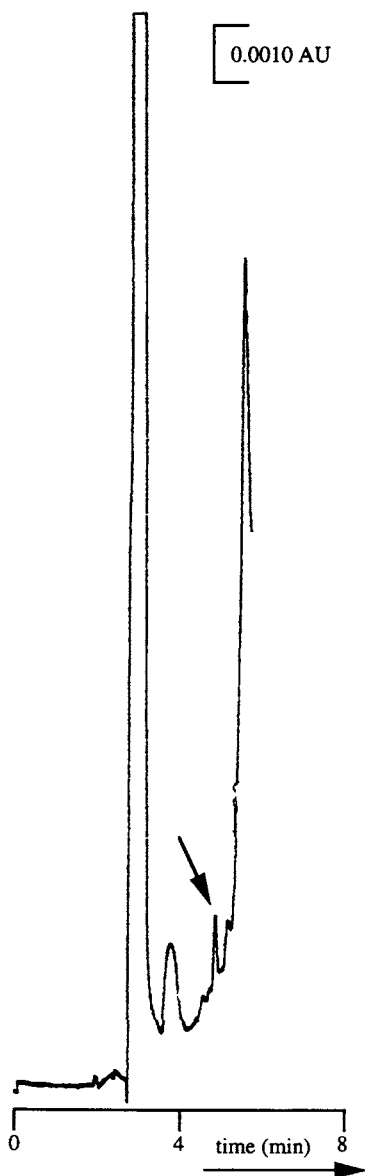
Fig. 8.9. Off-line SPE and LC/LC-UV (220 nm) of an SPE extract of a rain-water sample containing 0.05 μg/l of bentazone. For SPE procedure and LC/LC conditions, see Fig. 8.8B and Table 8.3.

feasible when combining off-line SPE with LC/LC-UV. A comparison between LVI on-line LC/LC-UV, and off-line SPE combined with LC/LC-UV for the trace analyses of triazines and some metabolites in water samples showed similar results. On-line LC/LC-UV favors speed of analysis, while a simple preceding off-line SPE-step provides LOQs in the range of 0.02–0.05 μg/l [22]. Another comparative study included the use of hydrophobic on-line SPE-LC (PC/LC) [28]. It appeared that, in comparison with LVI–LC/LC-UV and off-line SPE combined with LC/LC-UV, PC/LC favors sensitivity and speed of analysis at low levels (<0.1 μg/l) but its selectivity is considerably lower.

Off-line SPE and LC/LC-UV was applied successfully in an inter-laboratory study involving the determination of phenylurea herbicides spiked at levels of about 0.1 μg/l to drinking water, groundwater and surface water. The SPE procedure involved the sampling of 250 ml of water on a 500 mg C18 SPE cartridge preconditioned before use with 3 ml of methanol, 3 ml of acetone, 3 ml of methanol and 6 ml of LC-grade water. After sample loading, the cartridges were dried by passing air for 30 min and the analytes were desorbed with 2 ml of acetone. An aliquot of the acetone corresponding to 125 ml of sample was transferred into a tube and evaporated to dryness with a gentle stream of nitrogen. The residue was redissolved by adding 0.20 ml of acetonitrile followed by 1.8 ml of LC-grade water. The LC/LC-UV analysis (Fig. 8.10) of a groundwater sample containing monuron, monolinuron and diuron at levels of 0.15, 0.19 and 0.07 μg/l, respectively, effectively demonstrates the good performance of this approach in both selectivity and sensitivity.

In the case of acidic pesticides, the selectivity of RPLC-UV after off-line hydrophobic SPE is severely hampered by co-extracted humic acid interferences. For example, for the development of a screening method for the simultaneous determination of a group of chlorophenoxy acid herbicides (ε-values of around 8000 l/mol cm, and UV at 220 nm) in water, SPE of 50 ml of sample on a 100 mg C18 cartridge was applied in order to determine the analytes down to a level of 0.1 μg/l [29]. The RPLC-UV analysis of a SPE surface water extract, shown in Fig. 8.11A, clearly illustrates that because of the humic
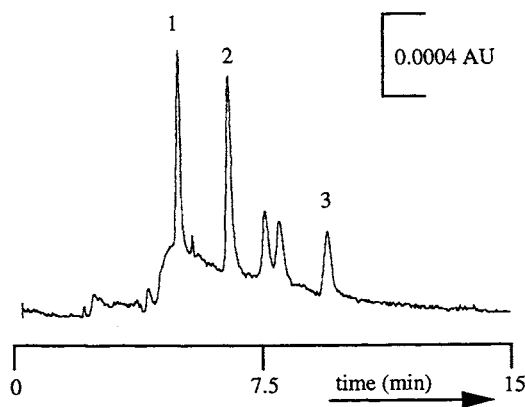
Fig. 8.10. Off-line SPE and LC/LC-UV (244 nm) of an SPE extract of a ground water containing (1), monuron 0.15 μg/l; (2), monolinuron, 0.19 μg/l; and (3), diuron, 0.07 μg/l. LC/LC conditions: C-1, 3 μm Microspher C18 (50 × 4.6 mm ID); C-2, 3 μm Microspher C18 (100 × 4.6 mm ID: M-1 and M-2, methanol–water (55:45%, v/v) both at 1 ml/min: injection of 150 μl of SPE extract (for procedure, see Text). Clean-up volume, 1.3 ml. Transfer volume 3.4 ml.

hump, as a result of the chromatographic behaviour, and the large transfer-volume (4.13 ml), LC/LC using two C18 columns is not suitable for this type of analysis. A large part of the humic interference could be removed efficiently with SPE procedures as outlined in Fig. 8.12. The SPE–silica procedure involving a phase-switch was first selected and, by means of column switching using a precolumn packed with Internal Surface Reversed Phase (ISRP) material (5 μm GFF-II), chlorophenoxy acids were analysed in all types of water samples [29]. An example of this approach for the determination of chlorophenoxy acids in a spiked surface-water sample (2 μg/l) is given in Fig. 8.11B. Apparently, the ISRP material acts as a Restricted Access Medium (RAM), enhancing significantly the separation between small-molecular analytes and large-molecular humic acid substances.
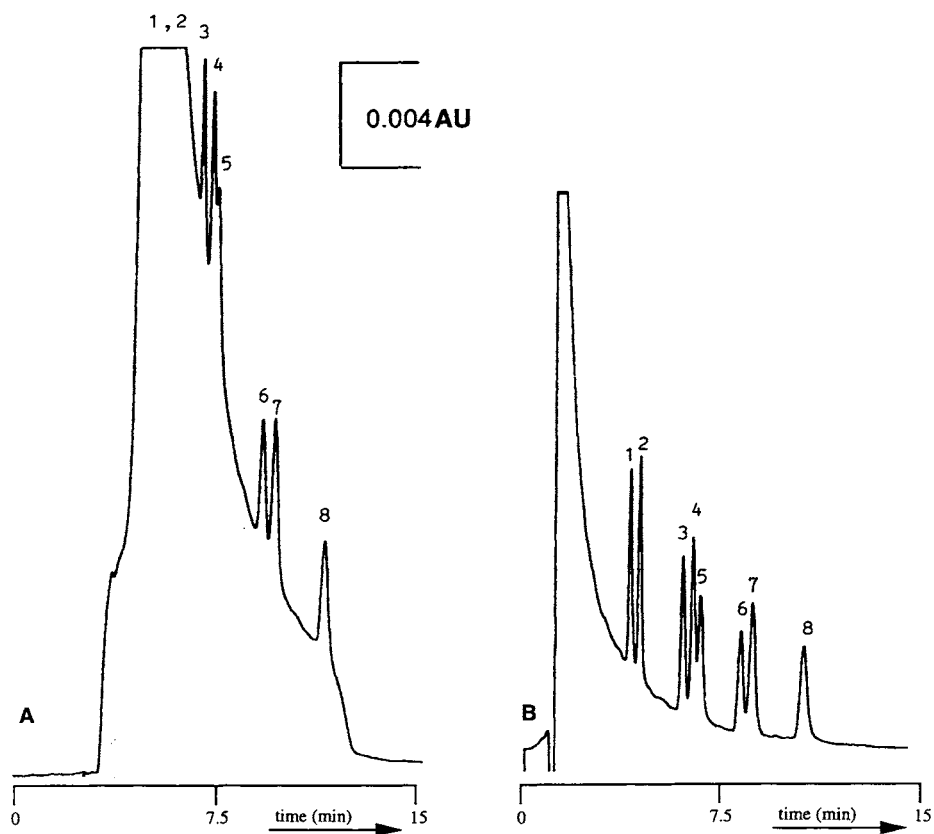
Fig. 8.11. LC/LC-UV (228 nm) vs. SPE-LC (228 nm) with ISRP precolumn for the analysis of an SPE C18 extract (SPE procedure, see Fig. 8.12) of a surface-sample spiked with eight chlorophenoxy acids at a level of 2.0 μg/l. (A) LC/LC with conditions, C-1 and C-2, 3 μm Microspher C18 (50 × 4.6 mm ID), M-1 and M-2, methanol–0.05% TFA in water (60:40%, v/v) at a flow rate of 1 ml/min. Clean-up volume, 2.37 ml. Transfer volume, 4.13 ml. (B) SPE-LC with conditions, C-1, 5 μm ISRP GFF-II (10 × 3 mm ID); C-2, 3 μm Microspher C18 (100 × 4.6 mm ID); M-1 methanol–0.05% TFA in water (5:95%, v/v) at a flow-rate of 1 ml/min; M-2, methanol–0.05% TFA in water (60:40%, v/v) at a flow-rate of 1 ml/min. Cleanup volume, 1.0 ml. Transfer volume, 0.50 ml. Pesticides: (1) 2,4-D; (2) MCPA; (3) 2,4-DP; (4) mecoprop (MCPP); (5) 2,4,5-T; (6) 2,4-DB; (7) MCPB; (8) 2,4,5-TP.
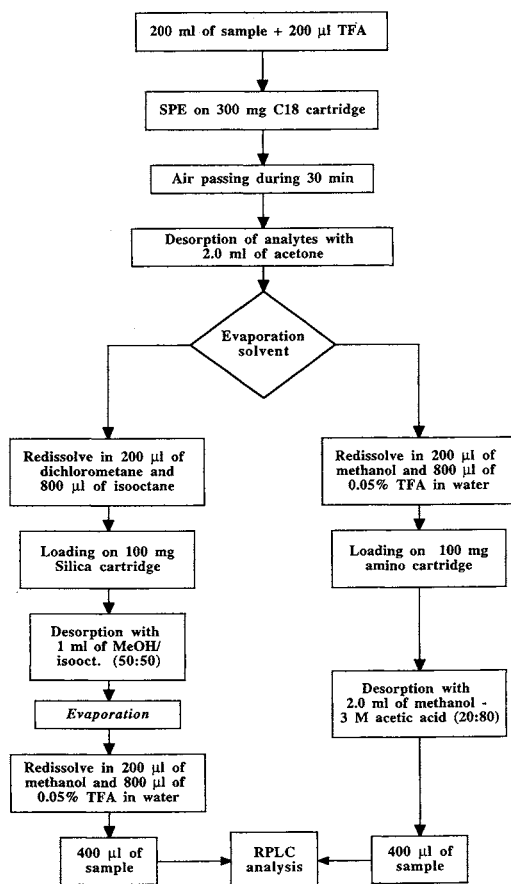
Fig. 8.12. Off-line SPE procedures for the determination of chlorophenoxy acids in water.

Unfortunately, the ISRP precolumns of different batches showed poor reproducibility in the required retention of the analytes and, hence, provided insufficient selectivity. The impairment could be recovered by using the more selective SPE clean-up on amino-bonded silica cartridges (see Fig. 8.12) in combination with LC/LC using two C18 separation columns. The SPE amino procedure provides a selective trapping of chlorophenoxy acids and was applied as a powerful clean-up in the analysis of chlorophenoxy acids in mammalian tissues [30].

A new development in improving RPLC separations between acidic pesticides and humic acid interferences in the analysis of environmental samples is the use of analytical RAM columns. Originally, these columns were developed for the on-line processing of body fluids such as serum and blood, providing retention for analytes but eluting large molecules such as proteins, in the void column volume [31–33]. Because of the large difference in molecular size between pesticides and humic substances the use of RAM columns seems to be attractive in this field of analysis. In our first approach, the use of ISRP precolumns confirmed this expectation [29] but, as mentioned above, their reprodu-

cibility appeared to be inadequate. This drawback was also observed in a study of the on-line processing of serum using RAM columns with column switching [34]. It appeared that the use of an analytical ISRP column distinctly more robust then a precolumn when used as C-1 in LC/LC–TSP/MS/MS in the on-line determination of β-agonists in serum [34]. As shown later, an analytical ISRP column was also applied successfully for the determination of mecoprop in soils. In a study concerning water samples, the use of a 150 × 4.6 mm RAM column packed with 5 μm Semi-Permeable-Surface (SPS) material as C-2 in LC/LC-UV enhanced considerably the selectivity, making it possible to trace bentazone and bromoxinil in difficult types of surface water samples, viz., ditch-water containing an excessive amount of interferences of high molecular weight, after an off-line SPE of 250 ml of sample on a 500 mg C18 cartridge [35].

In a recent comprehensive study [36], the applicability of several commercially available analytical RAM columns was investigated in LC/LC-UV for the trace analysis of acidic herbicides in water samples. Various LC configurations were studied, including the single-RAM column mode, and column switching (LC/LC) employing one RAM column in combination with an analytical C18 column, or two RAM columns. A group of acidic herbicides representing different chemical families was tested under both the SRM and MRM approach, by analysing SPE C18 extracts of reference water samples spiked with the analyte(s) at the 0.5–1.0 μg/l level and containing dissolved organic carbon (DOC) between 3 and 12 mg/l. The SPE was performed by percolating 250 ml of water sample, previously adjusted to pH 2.2 with hydrochloric acid, through a 500 mg C18 cartridge. After sampling, the cartridge was dried by passing air for 30 min. The analytes were desorbed with 2 ml of acetone and, after solvent evaporation, the residue was redissolved in 800 μl of methanol followed by 3.2 ml 0.05% TFA in water. Information on the types RAM of columns studied, and adequate coupled-column conditions, is given in Table 8.4.

As regards SRM, the combination of one analytical RAM and a C18 column, or the use of two different types of RAM columns sufficiently improved the resolution between the analyte and the humic interferences to allow trace analysis to be performed on the SPE extracts without a preceding cleanup.

Both ISRP and Hisep RAM columns provide a very efficient pre-separation between acidic analytes and humic interferences. However, these columns showed a separation mechanism different from C18, providing a large additional band-broadening and reversed elution order for some of the analytes. As can be seen in Fig. 8.13 this property, combined with the high retention capacity, makes Hisep rather poorly compatible with the second analytical column. The reversed elution counteracts the second separation, and the excessive band broadening of bentazone and bromxynil cannot be repaired with the second mobile phase.

In the case of ISRP, the distinctly lower retention capacity allows one to repair band broadening. This is nicely demonstrated in Fig. 8.14, showing the LC/LC-UV analysis of a water sample containing a DOC content of 12 mg/l and spiked with the analytes at the 0.5–1.0 μg/l level.

8.3.4 Applications to soil samples

Soil will contain more interferences than water, hence, clean-up will be even more important in the analysis of soil extracts, making coupled-column RPLC an attractive

TABLE 8.4

OVERVIEW OF LC/LC CONDITIONS USING ANALYTICAL RAM COLUMNS AND ALLOWING SINGLE (SRM) OR MULTI-RESIDUE (MRM) TRACE ANALYSIS OF ACIDIC HERBICIDES IN SPE EXTRACTS[a] OF DOC CONTAINING (6–12 mg/l) WATER SAMPLES

Method	Herbicides	C-1[b]; M-1[c]	C-2[b] and M-1[c]	Cleanup/transfer volume (ml)
SRM	Mecoprop	C18 (50 × 4.6 mm ID); 60%	SPS (150 × 4.6 mm I.D); 60%	3.6/1.0
		SPS (50 × 4.6 mm ID); 60%	Hisep (50 × 4.6 mm ID); 60%	4.0/1.0
		Hisep (50 × 4.6 mm ID); 60%	C18 (100 × 4.6 mm ID); 60%	3.5/1.0
		ISRP (50 × 4.6 mm ID), 40%	C18 (100 × 4.6 mm ID); 60%	5.0/1.0
SRM	Metsulfuron methyl	Hisep (50 × 4.6 mm ID); 40%	C18 (50 × 4.6 mm ID); 40%	2.8/0.8
		ISRP (50 × 4.6 mm ID); 40%	C18 (50 × 4.6 mm ID); 40%	2.2/1.0
MRM	Bentazone, bromoxynil	C18 (50 × 4.6 mm ID); 35%	SPS (150 × 4.6 mm ID); 40%	2.9/0.9
MRM	Bentazone, bromoxynil, metsulfuron-methyl, MCPA, mecoprop	ISRP (50 × 4.6 mm ID), 40%	C18 (50 × 4.6 mm ID); 60%	2.3/2.9
		ISRP (50 × 4.6 mm ID), 40%	SPS (50 × 4.6 mm ID); 55%	2.2./3.6
		C18 (50 × 4.6 mm I.D); 52.5%	SPS (50 × 4.6 mm ID); 52.5%	1.9/5.9

[a] Procedure: Load of 250 ml of water sample (brought at pH 2.2 with HCl) onto a 500 mg C18 cartridge. Desroption with 2 ml of acetone. After evaporation, redissolved in 0.4 ml methanol and 1.6 ml of 0.1 TFA in water.

[b] Packing materials: C18, 3 μm Microspher C18 (Chrompack); SPS, 5 μm SPS-5PM-S5-100-ODS (Regis); ISRP, 5 μm Pinkerton ISRP GFF-II-S5-80 (Regis); Hisep, 5 μm Hisep (Supelco).

[c] Modifier content in mobile phase consisting of acetonitrile–0.03 M phosphate (pH 2.4) (v/v).
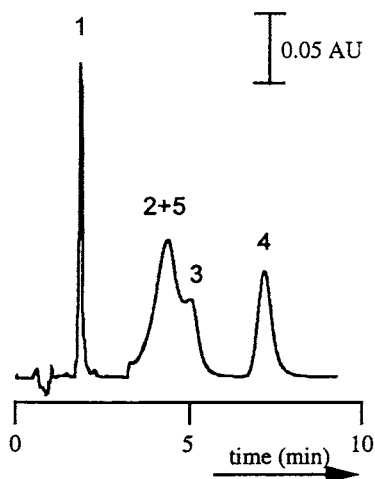
Fig. 8.13. Elution of acidic pesticides on a 5 μm Hisep RAM column (50 × 4.6 mm ID). Analytes: (1), metasulfuron-methyl; (2), bromoxynil; (3), bromoxynil; (4), MCPA; (5), mecoprop. LC conditions: mobile phase, methanol–0.05% TFA in water, pH 2.4 (55:45%, v/v) at a flow of 1 ml/min; injection, 300 μl of standard solution (4 μg/ml of each analyte) in methanol–0.05% TFA in water (20:80%, v/v); UV detection at 220 nm.
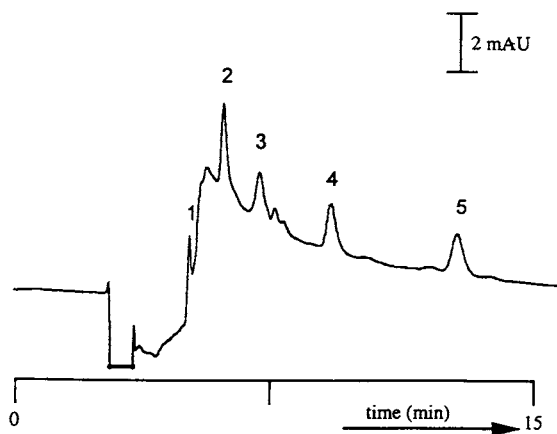
Fig. 8.14. LC/LC-UV (220 nm) employing two analytical RAM columns of 300 μl of extract obtained after SPE (for procedure, see Text) of a reference water sample containing 6 mg/l DOC and spiked with acidic pesticides at the 0.5–1.0 μg/l level. LC conditions: C-1, 5 μm ISRP GF-II (50 × 4.6 mm ID); C-2, 5 μm SPS-5PM-S5-ODS (50 × 4.6 mm ID; M-1, methanol–0.05% TFA in water (40:60%, v/v) at 1 ml/min; M-2, methanol–0.05% TFA in water (55:45%, v/v) at 1 ml/min; injection volume, 300 μl; cleanup volume, 2.2 ml; transfer volume, 3.6 ml. Analytes (and levels): (1) metasulfuron-methyl (0.5 μg/l); (2) bromoxynil (0.5 μg/l); (3) bentazone (0.5 μg/l); (4) MCPA (1.0 μg/l); (5) mecoprop (1 μg/l).

technique for performing efficient on-line cleanup. In a monitoring programme aimed at the occurrence of the more persistent pesticides, two SRMs were developed for the analysis of the pesticides pencycuron and fenpropimorph in soil.

A rather simple sample extraction–concentration procedure prior to LC/LC-UV has been developed. It consisted of an overnight standing extraction of 50 g of soil with 150 ml of acetonitrile. The extract is poured over a Büchner filter and rinsed with about 50 ml of acetonitrile. After volume adjustment to 200 ml with acetonitrile, 100 ml of extract is taken and concentrated to a volume of about 10 ml by rotating-film evaporation
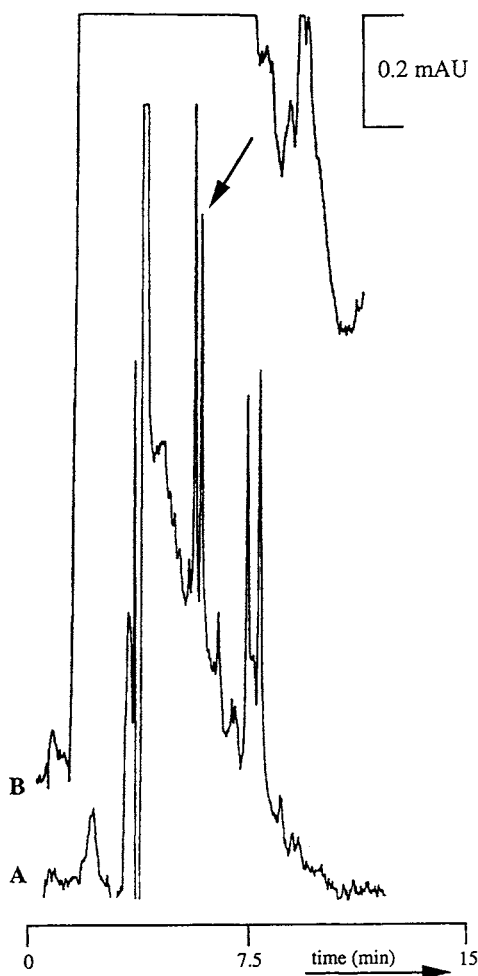
Fig. 8.15. LC/LC-UV (240 nm) of 100 μl of extract of a soil sample spiked with pencycuron at a level of 50 μg/kg. LC conditions (A): C-1, 5 μm Hypersil SAS (60 × 4.6 mm ID); C-2, 5 μm Hypersil ODS (150 × 4.6 mm ID); M-1, acetonitrile–water (40:60%; v/v); M-2, acetonitrile–water (70:30%, v/v); flow rates, 1 ml/min; clean-up volume, 3.00 ml; transfer volume, 0.50 ml. LC conditions (B): C-1 and C-2 connected in series without column switching and using mobile phase M-2.

at 40°C. The concentrate is then transferred to a 25 ml volumetric flask and made up with acetonitrile. Prior to LC analysis, 2 ml of extract and 2 ml of water are mixed and passed through a 0.20 μm filter. From this solution 100 μl is injected into the LC/LC system. The UV-detectability of both compounds is rather good, and their apolar nature provides a high degree of C18 retention (see Table 8.3). In such a case, the selection of a first column packed with C1-modified silica particle (5 μm Hypersil SAS) in order to reduce the analytes' hydrophobic retention, appeared to be favorable. In combination with a C18 column as C-2 and a mobile phase, M-2, with a higher eluotropic strength, a nice peak-compression could be obtained, significantly enhancing the sensitivity. The gain in selectivity provided by the developed LC/LC-UV procedure RPLC is shown in Fig. 8.15, which shows the analysis of soil spiked with 50 μg/kg of pencycuron.

For the determination of very polar analytes, such as ETU, the poor C18 retention imposes limitations on LC/LC-UV for trace analysis in difficult matrices such as hydrophobic soils. Thus, additional clean-up will be necessary for polar analytes. One can advantageously make use of the analytes' low C18 retention by applying a rapid SPE procedure, which was first applied to apple juice [37]. In this case, the application of SPE is reversed: the C18 cartridge traps the less polar components, while the very polar ones start to elute immediately. These analytes are then collected during sampling, thus providing some kind of a polarity cut-off filter. It must be mentioned that after the SPE step, LC/LC is still required to obtain sufficient selectivity. This SPE approach was applied successfully for the determination of ETU in soil samples, making it possible to analyse ETU to a level of at least 0.01 mg/kg [14].

Recently, it has been demonstrated that the use of an analytical RAM column in LC/LC-
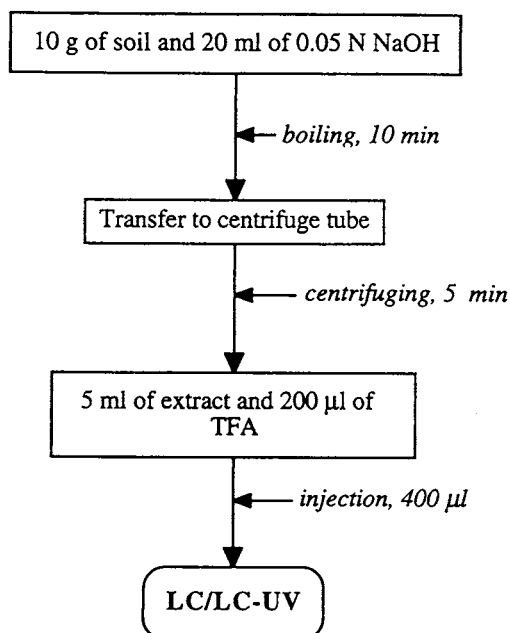
Fig. 8.16. Scheme of the analysis of mecoprop in soil.
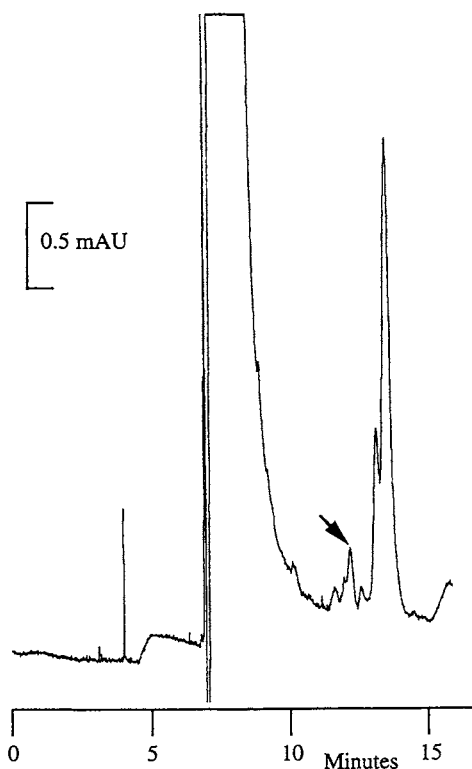
Fig. 8.17. LC/LC-UV (228 nm) employing a RAM analytical column as C-1, of 400 μl of extract of a soil sample spiked with mecoprop (MCPP) at a level of 20 μg/kg. Sample pretreatment, see Fig. 8.16. LC conditions: C-1, 5 μm ISRP GFF-II (50 × 4.6 mm ID); C-2, 5 μm Microspher C18 (50 × 4.6 mm ID; M-1, methanol–0.05% TFA in water (20:80%, v/v); M-2, methanol–0.05% TFA in water (55:45%, v/v); flow rates, 1 ml/min; clean-up time, 4.8 ml; transfer time, 1.6 ml.

UV is also favorable for the analysis of acidic compounds in soils [38]. For the screening of mecoprop in soils a LC/LC-UV method has been developed using a 50 × 4.6 mm ID RAM column packed with 5 μm ISRP (Pinkerton) as C-1. It appeared that, in comparison to an analytical C18 column, the ISRP column substantially improved the separation between acidic analyte and co-extracted humic substances. Under the selected LC/LC conditions, the soil extracts obtained after a pre-treatment procedure, as displayed in Fig. 8.16, could be analysed directly, allowing the determination of mecoprop in soils at a level of 0.02 mg/kg. The performance of this efficient approach is illustrated in Fig. 8.17, which shows the LC/LC-UV analysis of an extract of a soil spiked with mecoprop to a level of 0.02 mg/kg.

8.4 LC/LC WITH SELECTIVE DETECTION

The previous sections showed the universal and versatile aspects of LC/LC-UV in the

trace analysis of polar pesticides in environmental samples, leading to the production of many applications which use this technique. However, UV detection cannot always provide sufficient sensitivity and/or selectivity. A number of examples are given below of studies using LC/LC in combination with selective detection methods.

8.4.1 Fluorescence detection (FD)

The phosphinic acids, glufosinate and glyphosate are used widely in agriculture as non-selective contact herbicides. Because of their high polarity, amphoteric properties, and poor detectability, a methodology to determine these compounds at trace levels requires a large effort in the sample preparation steps such as extraction, concentration, derivatization and clean-up. A study on the determination of glufosinate in environmental water samples [38] demonstrated that the combination of precolumn derivatization with 9-fluorenyl methylchloroformate (FMOC-Cl) and LC/LC-FD improves considerably the sample throughput. The method uses a C18 column coupled to an ion-exchange amino column with a mobile phase of acetonitrile–0.05 M phosphate buffer (pH 5.5) (35:65%, v/v) for both columns. It must be emphasized that the clean-up performance here is obtained in a reversed way. Instead of removing an excess of early eluting interferences (S1, see Figs. 8.1 and 8.2) one makes use advantageously of the amphoteric property of FMOC–glyphosate. In this LC/LC approach the mobile phase ionizes the analyte, which will result in little C18 retention on C-1 and an adequate retention on C-2. The efficient clean-up is obtained by transferring an almost unretained small analyte containing fractions from C-1 to C-2. All interferences with more C18 retention, e.g. the excess of FMOC-OH is retained on C-1 and sent to waste by the rinsing mobile phase during the separation of the analyte on C-2.

It was established that LVI could be achieved by reducing the percentage of modifier (acetonitrile) of the sample after FMOC derivatization. For glufosinate, a four-fold dilution step with a borate buffer provides enrichment of the polar FMOC–glufosinate during LVI, up to 2 ml before breakthrough. After injection, the mobile phase provides that on the C18 column the analyte elutes as an almost unretained compound. This effect allows peak-compression so that a small transfer volume of approx. 250 μl transfers the analyte to the amino column. This favorable chromatographic behaviour is illustrated in Fig. 8.18, showing the establishment of clean-up and transfer conditions of a 2.0 ml injection, C-1, connected to the fluorescence detector. In this way, an efficient separation between the large excess of the FMOC reagent and the analyte is realized.

The value of the approach described was also investigated for the determination of glyphosate and its main metabolite, aminomethylphosphonic acid (AMPA), in environmental water samples [39]. It appeared that for glyphosate an 8-fold dilution step was necessary to avoid excessive band broadening of glyphosate–FMOC during LVI. Glufosinate, glyphosate, and AMPA could indeed be assayed simultaneously in aqueous samples at a level of 1 μg/l. An example of this approach is given in Fig. 8.19, showing the LC/LC-FD analysis of a surface-water sample spiked with three analytes at a level of 4 μg/l.

Single-residue methods focused on the analysis of one analyte, applying very accurately adjusted volumes for dilution, clean-up, and transfer, allowed the determination down to the 0.1 μg/l level. With single-residue methods, glufosinate, glyphosate, and AMPA were
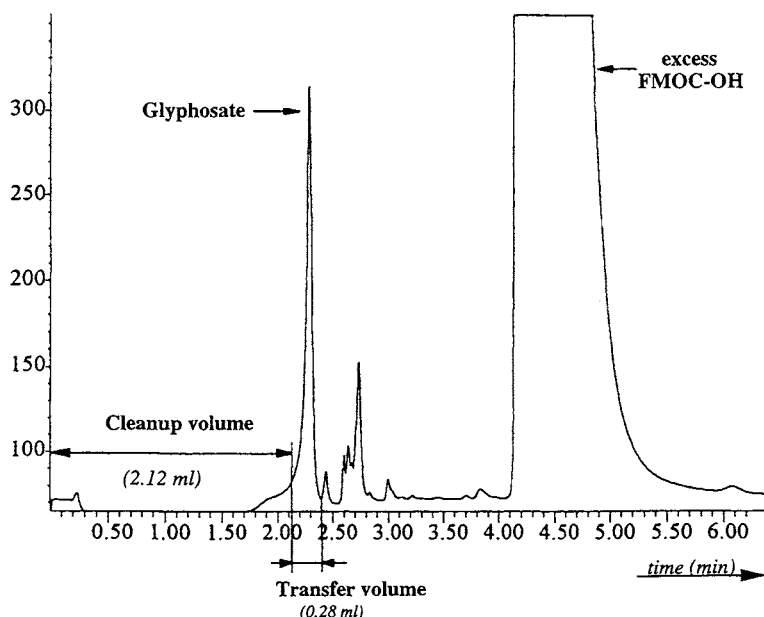
Fig. 8.18. Determination of volumes for clean-up and transfer for the analysis of glyphosate by the injection of 2.0 ml of a solution of glyphosate standard (0.4 μg/ml) obtained after FMOC derivatization and dilution on C-1, 5 μm C18 (30×4.6 mm ID), directly connected to the fluorescence detection ($\lambda_{ex} = 263$ nm, $\lambda_{em} = 317$ nm) [40].

successfully recovered from water samples at the 0.5–10 μg/l fortification levels, with a sample-throughput of at least 40 samples per day [40]. The same approach has been applied for the determination of glyphosate and AMPA in soil samples [41]. After extraction in alkaline media an aliquot of the extract is neutralized and processed with the procedures mentioned above. Depending on the type of soil, i.e. high- or low clay-content and organic matter, the limits of determination are 100 and 10 μg/kg, respectively, for both analytes.

8.4.2 Mass spectrometric detection (MS)

The use of LC with selective mass spectrometric detection (MS) for the trace-determination of pesticides and metabolites in the environment is a fast-growing technique. Concerning water samples, a large number of applications involving on-line and/or off-line techniques has been published and reviewed [42], indicating the suitability of the approach. At first sight, the hyphenation of LC/LC with selective MS detection would easily provide an overkill in separation power. However, for the determination of β-agonists in body fluids with LC/LC hyphenated to thermospray tandem mass spectrometry (LC/LC–TSP/MS/MS) the favorable features of LC/LC were clearly demonstrated [34,43]. For example, in order to avoid disturbances in TSP/MS/MS detection owing to changes in the pressure and flow-rate of the mobile phase, LC/LC is to be preferred over conventional LC. Furthermore, LC/LC with highly efficient separation columns allows
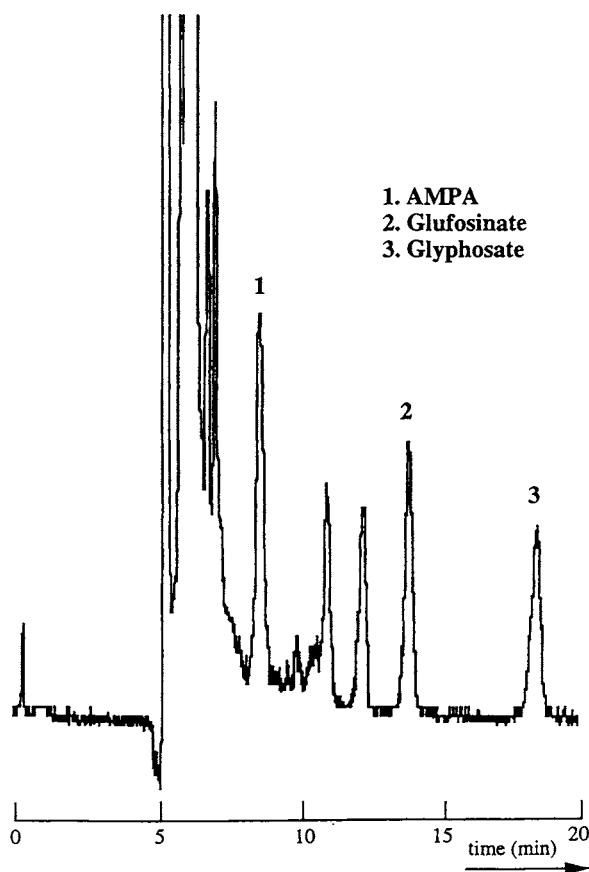
Fig. 8.19. LC/LC–FD ($\lambda_{ex} = 263$ nm, $\lambda_{em} = 317$ nm) of 2.0 ml of solution obtained after derivatization with FMOC [40] and 8-fold dilution with a borate buffer, of a surface-water sample spiked with the analytes at a level of 4 μg/l. LC conditions: C-1, 5 μm C18 (30 × 4.6 mm ID); C-2, 5 μm NH$_2$ (250 × 4.6 mm ID); M-1 and M-2, acetonitrile–0.05 M phosphate buffer, pH 5.5 (35:65%, v/v), both with a flow of 1 ml/min; cleanup volume, 2.21 ml; transfer volume, 0.53 ml.

large-volume sample injection for compounds with little C18 retention, without excessive band broadening. This enhances the sensitivity for the analyte, and on-line clean-up between the analyte and non-volatile compounds (e.g. inorganic salts). In conclusion, LC/LC-MS can provide very fast and selective methods. Recently, we investigated the potential of on-line LC/LC-MS for the determination of phenylurea herbicides in water samples. LC/LC-APCI/MS was carried out employing 10 ml LVI of sample, two columns (50 × 4.6 mm ID and 100 × 4.6 mm ID) packed with 3 μm C18, APCI-MS and optimized LC/LC conditions for clean-up (= removal of S1), separation, and time of analysis. As demonstrated by the ion chromatograms shown in Fig. 8.20 for the on-line LC/LC-APCI/ MS analysis of a surface water sample containing the herbicides at levels between 0.1 and 0.2 μg/l, this approach combines high selectivity and sensitivity with a high sample throughput.
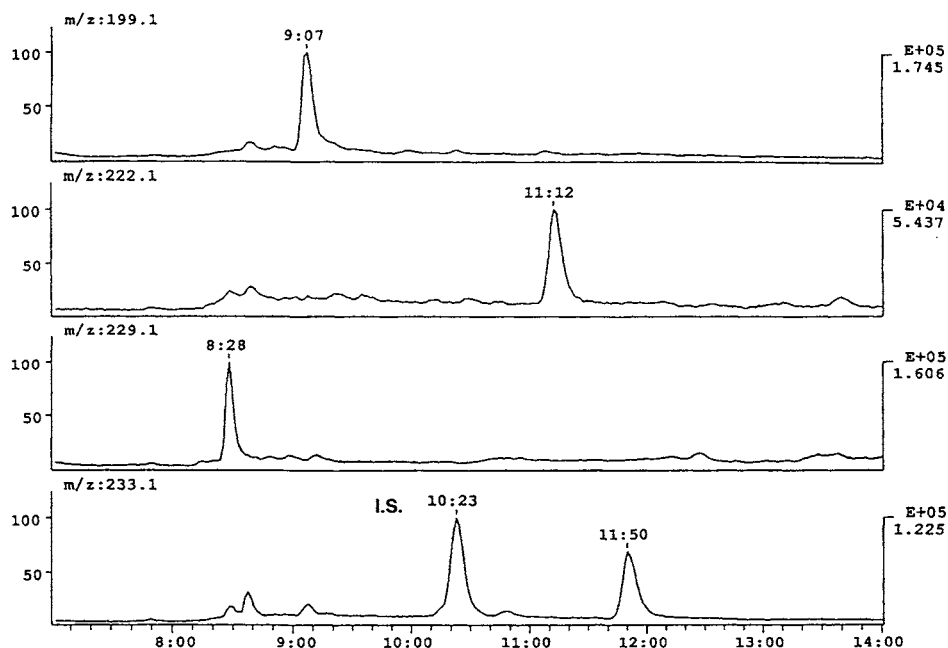
Fig. 8.20. Ion chromatograms of on-line LC/LC-APCI/MS of a surface water sample containing herbicides at a levels of about 0.1 μg/l. LC conditions: C-1, 3 μm Microspher C18 (50 × 4.6 mm ID); C-2, 3 μm Microspher C18 (100 × 4.6 mm ID); M-1, methanol–water (10:90%; v/v) during 15 min, followed by a gradient to methanol–water (60:40%; v/v) in 6 min; M-2, methanol–water (60:40%, v/v); flow rates, 1 ml/min; sample injection volume, 11 ml; clean-up volume, 22 ml; transfer volume, 6.0 ml. Compounds at retention times: 9.07 min, monuron (0.14 μg/l); 11.12 min, methabenzhtiazuron (0.21 μg/l); 8.28 min, metoxuron (0.23 μg/l); 11.50 min, diuron (0.12 μg/l); 10.23 min, fluormeturon, as internal standard (IS).

8.5. SYSTEMATIC METHOD-DEVELOPMENT

As outlined in Fig. 8.3, the development of coupled-column RPLC-UV methods starts with the selection of initial mobile and stationary phases, followed by some scouting experiments providing detection characteristics of the compounds to be analysed. It must be stressed that, from the point of view of efficiency, one should not optimize the method beyond a certain degree: the LC system must be kept as simple as possible. For example, if sample extracts are clean enough, column-switching can be omitted. However, as shown above, environmental trace analysis requires in most cases an efficient clean-up of sample or extract.

On the basis of trial-and-error experiments the LC/LC methods discussed above have been derived in a rather straightforward (see Fig. 8.3). However, in a few studies it appeared to be very difficult to find adequate LC/LC conditions for the separation between analytes and/or interferences. For such cases, sophisticated method-development procedures based on simulation and/or calculation were developed in order to avoid excessive experimental work [44–46].

The mobile phase system to be optimized in column switching is a (multi-) step-gradient elution over the first column (see Fig. 8.2). Step-gradient elution on both C-1 and C-2 can also serve as a good alternative for gradient elution since it shortens the run-time for analytes with large differences in retention [47]. As a first step towards the development of dynamic MRMs for pesticides with LC/LC a simulation programme was devised [44]. It was based on a diffusion model, and the main objective was an accurate prediction of retention times and peak volumes. As input, the program requires experimentally obtained retention data of the target compounds and, if possible, the interfering matrix peaks, described by the capacity factor, k, as a function of the mobile phase composition, φ, as well as the total plate-number of the column(s). The ln k/φ data were converted into second-order polynomial relationships. The program enables the analyst to search for suitable clean-up and transfer conditions in coupled-column RPLC. In this stage, no formal optimization criteria were formulated, and suitable conditions were found step-by-step with selected elution profiles, making the final choice of conditions dependent on the expert judgement of the analyst. Consequently, boundary conditions were formulated for development of MRMs using LC/LC [45].

Another step forward was the development of a computer spreadsheet for the automated optimization of step gradient elution conditions utilising optimization criteria [46]. The procedure is based on the use of derived analytical equations for the prediction of chromatographic data (retention and peak volume) of analytes eluting under isocratic, one-step and/or two-step gradient-elution profiles. Using the same input data as the ones used for the simulation procedure, the spreadsheet calculates for a given set of analytes the maximal resolution of the least efficiently separated pair of peaks in a three dimensional space defined by the eluotropic strength of the first and second mobile phase and the time at which the step-gradient takes place. In comparison to simulation, the developed calculation procedure enables a more rapid search for suitable conditions for on-line cleanup and separation of analytes using coupled-column RPLC, and is therefore highly productive and flexible.

In order to explain the steps applied in the method-development approach, one application [45] will be discussed in more detail. This study involved the determination in water of a heterogeneous group of nine polar pesticides including acidic, basic and neutral compounds. For these types of analytes the first parameter to be considered is the pH of the mobile phase. Initial experiments revealed that, at pH = 3.3, fair peak-shapes and reasonable retention could be obtained on a 100×4.6 mm ID column packed with 3 μm Microspher C18. The type and dimensions of this column are attractive, because after optimization of the separation one can easily apply two 50×4.6 mm ID columns with the same material to perform column switching. The RPLC behaviour of the compounds involved, shown in Fig. 8.21, reveals the large differences in polarity, and the crossing of several ln k/φ curves indicates that it will be difficult to obtain sufficient separation between some analytes.

On the basis of our earlier experience the selection of useful coupled-column separation conditions should include the four criteria given below.

1. The first-eluting analyte must have a retention which is at least twice as large as that of the unretained compound (similar to the SRM approach).
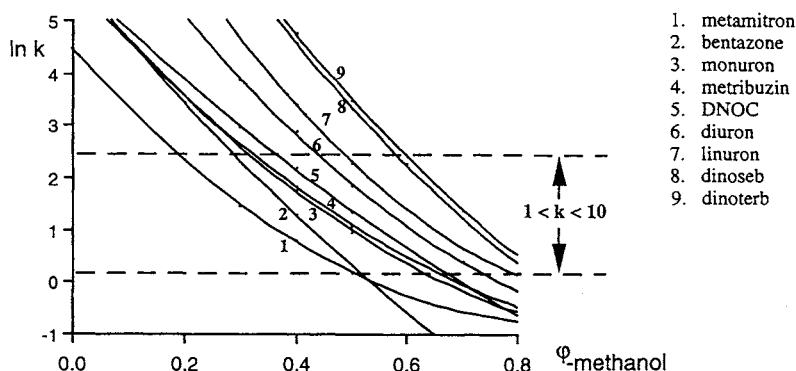
Fig. 8.21. Plots of ln k vs. φ of a group of various pesticides on 3 μm Microspher C18 column (50 × 4.6 mm ID) with methanol and 0.03 M phosphate buffer (pH 3.0) as mobile-phase constituents.

2. The total time of the chromatographic run should be kept relatively short (for sensitivity and sample throughput).
3. The resolution (Rs) between two adjacent peaks must be at least 1.2 to prevent problems from UV-wavelength switches and/or changes in the mobile-phase compositions.
4. The number of steps during gradient elution should be minimized, to reduce baseline distortions and the complexity of the LC system.

An interpretation of the ln k/φ curves (Fig. 8.21) can already provide the outlines for the method to be developed. Criteria 1 and 2 largely determine the applicable mobile-phase composition(s). In terms of retention, criteria 1 and 2 can simply be looked upon as $1 < k < 10$, thereby more or less determining the eluotropic strengths of the subsequent step gradient. The duration of a single mobile-phase composition is governed by criterion 3. The plots nicely illustrate the necessity of (step) gradient elution. Selecting the separation power of two 50 × 4.6 mm ID columns ($N = 10\,000$ for monuron), the computer programme was used to search for conditions meeting the criteria 1–4. Fig. 8.21 indicates that a mobile phase containing about 30% methanol can be used for the elution of the first analyte (metamitron), providing adequate clean-up (criterion 1) and sufficient separation from bentazone. However, the length of this (first) step is limited because of the poor resolution between monuron and metribuzin. For these analytes, and DNOC, elution with a mobile phase containing about 50% methanol will be more appropriate. Finally, an eluent containing about 70% methanol will provide proper elution and separation of diuron, linuron, dinoseb and dinoterb. Using this information, a two-step gradient meeting the four separation criteria was found rapidly with computer simulation. The good performance of this approach is given in Fig. 8.22, showing the good correspondence of a simulated and experimental chromatogram of an adequate two-step gradient elution.

However, during analysis at low levels it appeared that because of baseline distortions, a two-step gradient elution profile was inconvenient, and it also remains unclear whether criterion 4 is met. Meeting criteria 1 and 2, the selection of a suitable one-step gradient elution based on a graphical interpretation becomes less obvious, and requires large
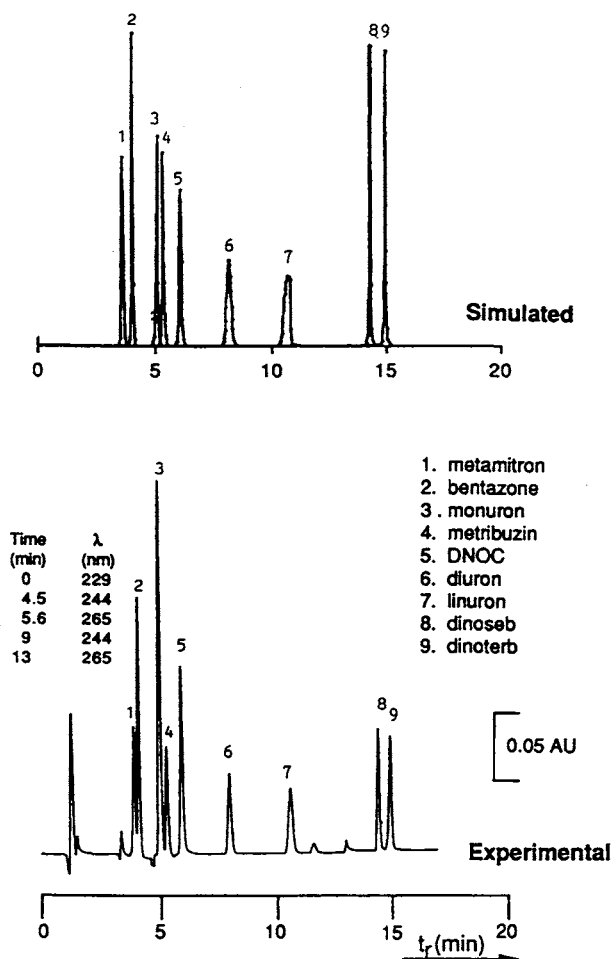
Fig. 8.22. Comparison of simulated and experimental chromatograms employing a two-step gradient elution (for conditions, see Fig. 8.23) for the optimized separation of nine polar pesticides (see Fig. 8.21).

numbers of chromatogram simulations. Hence, from both practical and chromatographic points of view, optimization should preferably start with the search of a suitable one-step gradient-elution profile, meeting criterion 4. For this requirement, the optimization procedure *OPTIME Version 3.1* described in reference [46] is very convenient. *OPTIME Version 3.1* is based on the use of analytical equations for the prediction of chromatographic data (retention and peak volume) of analytes eluting under one- or two-step-gradient (LC/LC) conditions. The procedure uses a constructed worksheet (Microsoft Excel) and can be applied as a calculation and/or an optimization procedure.

An example worksheet including input and output ranges, is shown in Fig. 8.23. The first five columns correspond to the calculation procedure, and show the flexibility and speed of this approach. Changing one of the LC parameters immediately updates the

Prediction of retention in step-gradiënt HPLC.   OPTIME V3.1   Copyright © RIVM 1992

LC parameters			No.	Analytes	tr	σ	Rs	Range of optimisation parameters			
Ø_start	28	%	1	metamitron	3.93	0.026	2.69		Min.	Max.	Step
Ø_step[1]	53	%	2	bentazone	4.21	0.026	2.69	tr	2	20	- min.
time[1]	2	min.	3	monuron	5.23	0.040	1.55	Ø_start	15	60	5 %
Ø_step[2]	70	%	4	metribuzin	5.49	0.044	1.55	Ø_step[1]		70	5 %
time[2]	9.5	min.	5	DNOC	6.19	0.052	3.66	time[1]	2	10	1 min.
gradiënt	2x		6	diuron	8.24	0.077	6.75	Ø_step[2]			%
			7	linuron	10.73	0.107	6.75	time[2]			min.
to	1	min.	8	dinoseb	13.02	0.056	2.56	one Yes		two No	
length column	100	mm.	9	dinoterb	13.63	0.064	2.56	Responsvalue	1.55		
volumes :											
column	1000	µl									
valve + loop	280	µl									
tubing	80	µl									
0.5 HETP	0.008	mm.									
flow	1000	µl/min.									
td	0.08	min.									
µ	100.0	mm/min.						Optimise step-gradient			

SMG

Fig. 8.23. Display of the input- and output screens of the calculation and optimization procedure *OPTIME version 3.1*. For further explanation, see Table 8.5 and Text.

chromatographic information (retention, peak volume and resolution) of the analytes. The optimization parameters displayed in the last part of Fig. 8.23 are described in Table 8.5. The optimization procedure, which is summarized in Fig. 8.24, is activated by the *Optimise step-gradient* compartment displays. After calculation, the found optimum (maximal resolution of the least efficiently separated pair of peaks) as the *Response value*. The procedure finds the optimum within the defined whole chromatographic range. However, information about alternative optima can be useful in experimental work. Therefore, as shown in Tables 8.6 and 8.7, the optimization procedure also generates on a separate work sheet a report of the best ten maximum responses with corresponding LC conditions and

Experimental determination retention behaviour of the analytes (plots of ln k vs φ) and N of columns

OPTIME V3.1

Automated optimisation coupled-column RPLC conditions

One-step gradient elution → Input ranges

tr-min: 2 min
tr-max: 20 min
φ–start: 15 -60%
φ–step[1]: 2.5%
time[1]:   2-10 min (0.25 min)

Report (Table 6)

Two-step gradient elution → Input ranges

tr-min: 2 min
tr-max: 20 min
φ–start: 15 -60%
φ–step[1]: 5%
time[1]:   2-10 min (0.5 min)
φ–step[2]: 80% (step 5%)
time[2]:   12 min (0.5 min)

Report (Table 7)

Fig. 8.24. Scheme of optimization procedure using *OPTIME version 3.1*.

TABLE 8.5

DESCRIPTION OF PARAMETERS USED IN *OPTIME VERSION 3.2* OPTIMISATION PROCEDURE

Parameter	Description
tr_{min}	Mininmal time of the first eluting analyte (clean-up)
tr_{max}	Maximal time of the last eluting analyte
φ_{start}	Min. and max. % methanol of mobile phase 1 (M-1)
Time[1]	Min. and max. time of the solvent switch 1
$\varphi_{step[2]}$	Min. and max. % methanol mobile phase 3 (M-3)
Time[2]	Maximal time of solvent switch 2

TABLE 8.6

REPORT OPTIME V3.1 USING A ONE-STEP GRADIENT ELUCTION[a]

	1	2	3	4	5	6	7	8	9	10
Min. Rs.	2.13	2.18	2.21	2.22	2.25	2.26	2.28	2.29	2.34	2.4
φ_{start}	37.5	37.5	45	37.5	35	37.5	45	37.5	45	45
$\varphi_{step[1]}$	60	62.5	62.5	65	62.5	67.5	65	70	67.5	70
Time[1]	7.25	7.25	4	7.25	9	7.25	4	7.25	4	4
$\varphi_{step[2]}$										
Time[2]										
tr_{min}	3.634	3.634	2.667	3.634	4.124	3.634	2.667	3.634	2.667	2.667
tr_{max}	19.2	16.76	13.31	14.92	18.55	13.53	11.52	12.47	10.16	9.131

00:33:21 (hh:mm:ss) calculating 2897 combinations

[a] For conditions, see Fig. 8.24.

TABLE 8.7

REPORT OPTIME V3.1 USING A TWO-STEP GRADIENT ELUTION[a]

	1	2	3	4	5	6	7	8	9	10
Min. Rs.	2.23	2.23	2.24	2.25	2.26	2.28	2.28	2.28	2.4	2.4
φ_{start}	45	45	45	45	45	45	45	45	45	45
$\varphi_{step[1]}$	70	65	65	65	65	65	65	65	70	70
Time[1]	4	4	4	4	4	4	4	4	4	4
$\varphi_{step[2]}$	75	75	80	75	75	70	75	80	75	80
Time[2]	7	7	9.5	7.5	8	4.5	8.5	10	7.5	7.5
tr_{min}	2.667	2.667	2.667	2.667	2.667	2.667	2.667	2.667	2.667	2.667
tr_{max}	8.845	9.576	11.02	9.884	10.19	9.325	10.5	11.4	9.03	8.97

09:27:06 (hh:mm:ss) calculating 49751 combinations

[a] For conditions, see Fig. 8.24.

Table 8.8

COMPARISON OF CHROMATOGRAPHIC DATA OF THE TESTED ONE-STEP GRADIENT ELUTION ON A 100 × 4.6 mm ID 3 μm C18 COLUMN[a]

Pesticide	Experimental		Calculated		Simulated	
	tr_{min}	σ_{min}	tr_{min}	σ_{min}	tr_{min}	σ_{min}
Metamitron	3.20	0.060	3.24	0.041	3.24	0.042
Bentazone	4.70	0.055	4.69	0.059	4.69	0.060
Monuron	6.80	0.080	6.74	0.085	6.74	0.085
Metribuzin	7.30	0.085	7.30	0.092	7.29	0.092
DNOC	8.70	0.030	8.99	0.030	9.01	0.030
Diuron	9.60	0.045	9.95	0.038	9.97	0.038
Linuron	10.70	0.050	10.88	0.047	10.90	0.042
Dinoseb	13.90	0.075	14.02	0.084	14.04	0.081
Dinoterb	15.00	0.075	15.04	0.097	15.06	0.093

[a] Time, 7.5 min with 40% MeOH, then 65% MeOH in 0.03 M phosphate buffer (pH 3.2).

updated retention times. As regards the production of chromatographic data and optimal separation conditions, the results obtained by *OPTIME Version 3.1* were in good agreement with the simulation procedure and experimental verification [46]. An example of chromatographic performance is given in Table 8.8, which emphasizes the usefulness of this tool in method-development.

8.6 CONCLUSIONS

LC/LC with UV detection is a versatile tool for the determination of polar and moderately polar organic pesticides in environmental samples. The work accomplished in this field of analysis demonstrates the favorable aspects of this technique such as the considerable enhancement of selectivity, sensitivity, and speed of analysis. For many polar pesticides with favorable RPLC-UV properties, sub-μg/l detection limits in aqueous samples are easily attainable in less then 10 min.

Hydrophobic LC/LC covers a wide range of pollutants to be determined in both multi- and single residue methods. A systematic approach to method-development renders the technique an important means for the operation of flexible monitoring programmes.

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Chapter 9

Liquid chromatographic and biorecognition techniques for the determination of phenols and their substituted derivatives in water samples

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CONTENTS

9.1 INTRODUCTION

   In all natural environments there are numerous biological interactions which are responsible for a variety of reactions - both ones known to man, and presumably unknown ones - which may be essential or hazardous to life. There is great interest in understanding basic mechanisms of diseases that are, or might be, linked to environmental factors which are, in some cases, shown very clearly in toxicity studies. Alternatively, although this is still unknown, these factors may in combination have multi-factorial effects and may affect human health. Interdisciplinary research collaborations may show these to include inflammatory disorders of one or several organ systems, as well as diseases related to dysfunctions of the immune system. Much attention is given to environmental areas which are of particular importance in elucidating the toxicity aspects of specific hazardous chemicals upon occupational exposure, as well as in everyday life from a common daily food intake.

   Phenolic compounds of environmental interest originate from a wide variety of industrial sources such as the plastics and dye industries, and particularly from wood-pulp

processing. Phenolic compounds are also generated from biodegradation processes in nature, such as decomposition products from humic substances which represent a large and very broad range of polymeric molecules, as well as from tannins and lignins. Additionally, chlorinated organophosphorus-, and phenoxy acid herbicides and pesticides generate chloro- and nitro- substituted phenols, respectively. Phenols and their substituted derivatives are generally toxic and can be linked directly to toxicological effects on humans. For example, many pesticides are used in the agricultural environment, and some may have the potential to disrupt reproductive or endocrine functions. Rawlings et al. found that both pentachlorophenol and triallate caused a significant increase in severity of oviductal intraepithelial cysts in ewes [1]. Data suggest that several currently used pesticides could influence serum concentrations of reproductive and metabolic hormones, particularly those of thyroxine, the major secretory product of the thyroid gland and a principal regulator of metabolism. Chlorophenols are present as pollutants in the aquatic environment as a result of degradation of pesticides and insecticides. Many of the phenols listed in the priority pollutants list are highly toxic and carcinogenic.

Phenols, and especially chlorophenols, are already toxic at a concentration of a few μg/l, and are also persistent. Consequently, many phenolic compounds are listed in the European Union (EC) directive 76/464/EEC, [91] which lists dangerous substances discharged into the aquatic environment, and in the USA where the US Environmental Protection Agency (EPA) list of priority pollutants also contains eleven phenolic substances [92–94]. The maximum amount of phenolic compounds allowed in surface waters by the EEC 75/440 directive is in the range of 1–10 μg/l, depending on the treatment required [95]. The official EPA analytical methodology in the USA includes acidification of the sample, followed by a liquid–liquid extraction using dichloromethane, to give an extract which is quantified by GC with electron capture or mass spectrometric detection.

The increasing human exposure to toxic influences in modern society, both in working environments and intake via the food chain, puts high demands on the development of analytical technologies and methodologies to ensure a safe and high quality of life. Our environment contains a great variety of infectious pathogens, and these toxic compounds can cause severe diseases. The site of infection and the type of pathogen largely determine which immune responses will be effective. Any immune response involves first, recognition of the pathogen or other foreign material and, secondly, mounting a reaction against it in order to eliminate it. The different types of immune response fall into two categories - the non-adaptive immune response and the adaptive immune response. The important difference between these two is that an adaptive immune response is highly specific for a particular pathogen. The close link between the environmental area and the biomedical understanding of the immune system which protects us from pathogens is clearly becoming an area of increasing scientific importance.

Although the toxicity of chlorophenols is generally established, the toxicity of chloroguaiacols and chlorocatechols towards aquatic organisms has been studied less extensively. Different phenolics do not behave in the same way. The ortho-substituted 2-chloro-, 2,6-dichloro-, and 2,4,6-dichloro-phenols were less toxic to fish cells than were the other isomers having the same number of chlorine atoms. Phenols, especially chlorophenols and chlorocatechols, are of importance in giving odour to river- and drinking waters. It has also been stressed that there is a need to analyze the different transformation products of all these compounds in water. The toxicity of phenol has been estimated to

vary between a few μg/l and mg/l, but it is reported that for certain species the highest permissible concentration of phenols cannot exceed 1 μg/l. In conclusion, there is interest from the point of view of toxicity regarding the xenobiotic exposure of humans to environmental and drinking waters: this is also indicated by the maximum allowable phenol concentration of 0.5 μg/l.

Another area of growing interest and importance relates to endocrine disrupters. Endocrine disrupters released into the environment can have adverse effects, with severe medical consequences to the nervous system and to the hormonal and other biologically regulating mechanisms in mammals. Oestrogen receptors represent a target area of great interest and concern. Both the α-receptors and β-receptors are known, and have been identified. The mechanism of the binding of compounds to the DNA, which regulates a number of important functions, is still to be elucidated. The affinity for other biomolecules might be higher in many cases than that of the α-, and β-receptor complex formation. The β-receptor probably has a function totally different from the α-receptor, that is mainly involved in replication.

In recent years there has been an increasing concern regarding non-ionic surfactants, which have phenolic structures. Compounds with surfactant skeletons find application in industrial processing technology and science, with major usage in detergents. Among these, non-ionic surfactants (NIS) possess specific properties, including relative ionic sensitivity and sorptive behavior, that makes them highly suited for use wherever interfacial effects of detergency such as (de)emulsification, (de)foaming, dispersion, or solubilization can enhance product- or process performance. NIS mainly consist of alcohol ethoxylates and alkylphenol ethoxylates. There is a continuous increase in the use of these chemicals in industry, both in Europe and in the USA, in total reaching amounts of 750 000 tons/annum [2].

9.2 SAMPLING STRATEGIES FOR WATER ANALYSIS USING MEMBRANE-BASED ANALYSIS

Sampling is often thought to be the most critical step in the whole analytical procedure. The whole analysis of the compound is dependent on a proper sampling step. Aspects such as the place and time, frequency, and equipment used for the sampling should be considered. Additionally, the preservation of the sample should be correct when it is transported from the sampling place to the laboratory.

Normally, filtration is used as a first step in many analytical methods - without considering the loss of reproducibility from losses caused by membrane interactions in the filtration. One important and widely used sample preparation method is based on the use of membranes under pressure [3–6], with dialysis which may have free diffusion [7] or be electrically driven [8]. By definition, a membrane is a selective semi-permeable barrier between two phases, the donor and the acceptor. The development of methods for automated on-line sampling and sample clean-up techniques in natural waters provides an important tool for enabling this control, and is therefore gaining increasing interest [3–6]. This also allows on-site measurements to be carried out along water-streams and to be used for early alarm systems. Membrane-based filtration techniques such as cross-flow or tangential-flow methods, and the use of hollow fibers, commonly provide rapid and effi-

cient sampling. A membrane filtration step provides a powerful way to eliminate solids, colloids, and other particulate matters in water samples, which normally cause clogging and column saturation in continuous monitoring.

Methodologies using supported liquid membranes (SLM) have been developed for the determination of 4-chlorophenols, 2,5-dichlorophenol, 2,4,5-trichlorophenols, 2,3,5,6-tetrachlorophenol and pentachlorophenol [9]. The method was based on an SLM extraction connected on-line to the CLC system. The SLM technique utilizes a porous PTFE membrane, which is impregnated with an organic solvent forming a barrier between two phases. This permits selective extraction with extraction efficiencies reaching down to the sub-μg/l level with a sampling time of 30 min. Sensitivities higher by orders of magnitude can be obtained by increasing the sampling time [10]. SLM was also applied to the sampling of phenolic acids in the irrigation waters from a closed hydroponic tomato culture with circulating nutrient solutions [10].

We have developed an on-line cross-flow filtration coupled to column liquid chromatography for the determination of some phenols in environmental waters [5]. The sampling unit (Waters™ Filter/Acquisition Module) is based on tangential flow filtration, in conjunction with column liquid chromatography.

The transport of solutes through the membrane barrier occurs because of a pressure difference, ΔP, that is built up across the membrane during pumping of a solution in the cross-flow mode. This pressure depends on the solution feed rate, the position of the built-in restrictor, and the dimensions of the outlet tubings.

The flux through a micro-filtration membrane can be expressed as

$$ J = \frac{\Delta P}{\eta \cdot R_{tot}} \tag{1} $$

where J = flux, (P = pressure, R_{tot} = total resistance of the filtration system and η = viscosity.

The goal was to obtain filtrates as clean as possible, to give sample chromatograms with minimal influence from matrix interferences. This would result in determinations with better precision and accuracy, and simplify the determination of polar analytes. We have been able to show that a proper choice of membrane, and membrane–sample interactions can circumvent fouling, concentration polarization, and other undesired sampling effects which make solute quantification impossible. Unpredictable and varying recovery values for phenols are then obtained owing to matrix components such as humic substances and other interferents which appear as a chromatographic front in HPLC–UV analysis.

The characterization and selectivity of planar cross-flow filtration membranes of 47 mm diameter for the sampling and sample clean-up of environmental waters containing phenols and high levels of humic substances were investigated. Seven membranes, both cellulosic- and polymer types were investigated using samples with 10–80 mg/l of humic acid. The different types investigated were: HAWP04700, Cellulose Esters (0.45 μm pore size) and GVWP04700, PolyVinylideneDiFluoride (PVDF) (0.22 μm) purchased from Millipore (Bedford, MA, USA), and further A045047A, Cellulose Nitrate (0.45 μm); C045A047A, Cellulose Acetate (0.45 μm); J050A047A, Teflon, 0.5 μm; P13013 Poly-Carbonate (PC) screen membrane(0.2 μm) and, finally, 83052, Nylon 4–6- (0.45 μm) [5]. Compounds of environmental concern such as 4-nitrophenol and 4-chlorophenol were
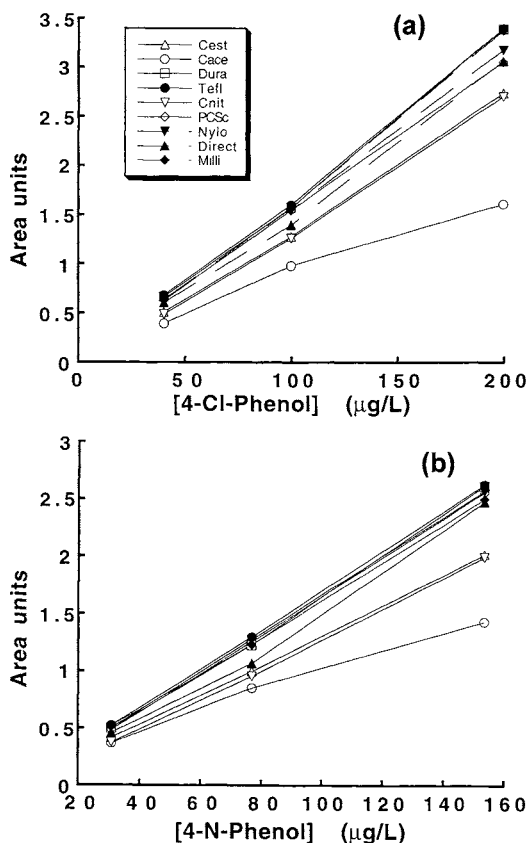
Fig. 9.1. Calibration curves for: (a), 4-chlorophenol; (b), 4-nitrophenol, and using a 10 mg/l humic acid matrix with various membranes.

chosen as model compounds and the respective calibrations with the various membranes are shown in Fig. 9.1a,b.

The repeatabilities and stabilities using nylon and PVDF membranes were evaluated, as well as flux variances, with analyte recovery values of around 100% in a CFF–LC–UV system for real surface water from a local creek, with a LOD of 1–5 μg/l ($S/N = 3$) using a 100 μl injection volume without using a SPE-step. Chromatographic separations of some phenols and triazines with the system described are shown in Figs. 9.2 and 9.3 for a mixture in water alone, and in humic-containing water, before and after filtration, respectively. To obtain more information about the membrane fouling processes, scanning electron microscopy (SEM) was used for a thorough investigation of cellulose acetate and nylon membranes. A sample solution of 40 mg/l humic acid and 40 μg/l pesticides was filtered for 0 h (flush of Millipore water), 0.5, 3, and 16 h, respectively. Figs. 9.4a,b and 9.5a,b illustrate the fouling process on a fresh membrane and one that has been exposed to humic-containing waters on a cellulose acetate, and a nylon membrane, respectively.

Good operational stability of the current set-up was shown in continuous on-line cross-flow filtration coupled to column liquid chromatography for the determination of phenols
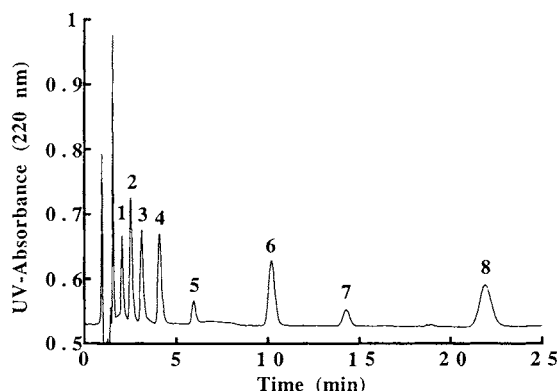
Fig. 9.2. Separation of analytes at 40 μg/l using millipore water with a pesticide mixture at 40 μg/l, after cross-flow filtration using cellulose acetate membrane. The chromatographic peaks represent: (1), hydroxysimazine; (2), desisopropylatrazine; (3), hydroxyatrazine; (4), desethylatrazine; (5), 4-nitrophenol; (6), simazine; (7), 4-chlorophenol; (8), atrazine.

in environmental waters. With this system, the guard column was exchanged every two weeks and the lifetime of the analytical column with these complex matrices was estimated to be around six months.

9.3 SEPARATION TECHNIQUES AND DETECTION PRINCIPLES FOR THE DETERMINATION OF PHENOLS

Phenolic compounds evince increasing interest owing to the increasing awareness of their toxicity, and the knowledge gained of the formation of transformation products from the various pesticides. CLC is by far the most common separation technique, followed by GC. The total analytical coverage of all phenolic compounds in environmental samples
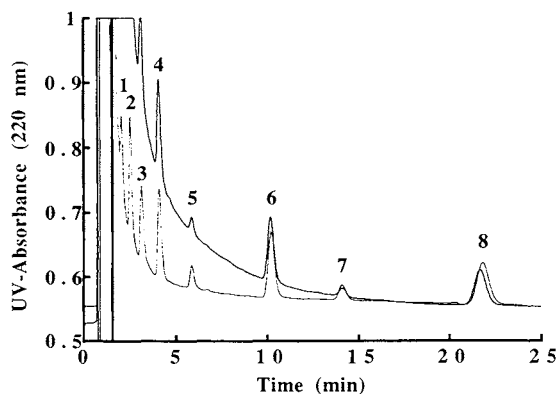
Fig. 9.3. Same conditions and sample mixtures as in Fig. 9.2, but with 40 mg/l of humic acid: upper, without membrane filtration; and lower, with nylon membranes. The chromatographic peaks represent: (1), hydroxysimazine; (2), desisopropylatrazine; (3), hydroxyatrazine; (4) desethylatrazine; (5), 4-nitrophenol; (6), simazine; (7), 4-chlorophenol; (8) atrazine.
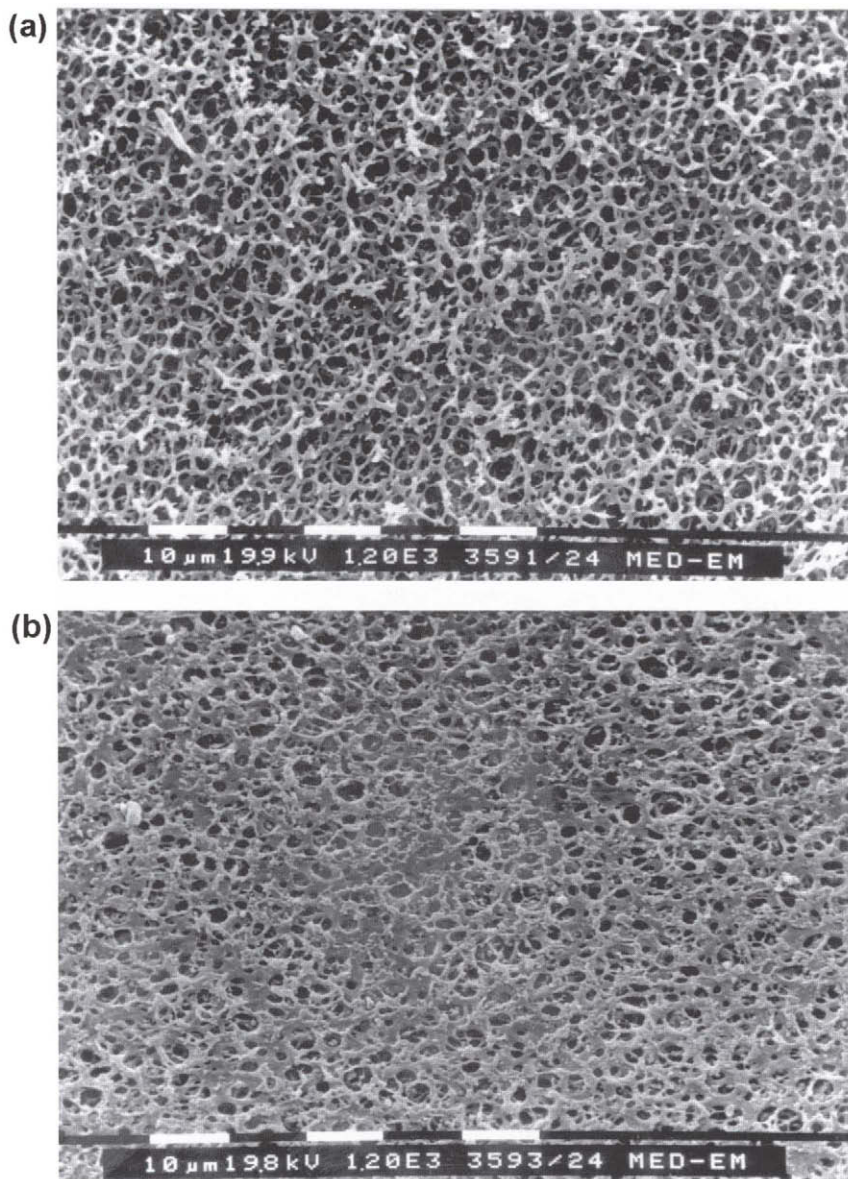
Fig. 9.4. Scanning electron micrographs of microfiltration membranes after cross-flow filtration of 40 mg/l humic acid containing pesticide samples: (a), fresh cellulose acetate membrane; and (b), after 16 h of use.

based on CLC-methodologies favors the use of reversed-phase gradient separations with mobile phases containing methanol or acetonitrile as organic modifier. The classical work by Horvath's group at Yale [11,12] is still highly relevant and various optimizations from these papers are still important today.
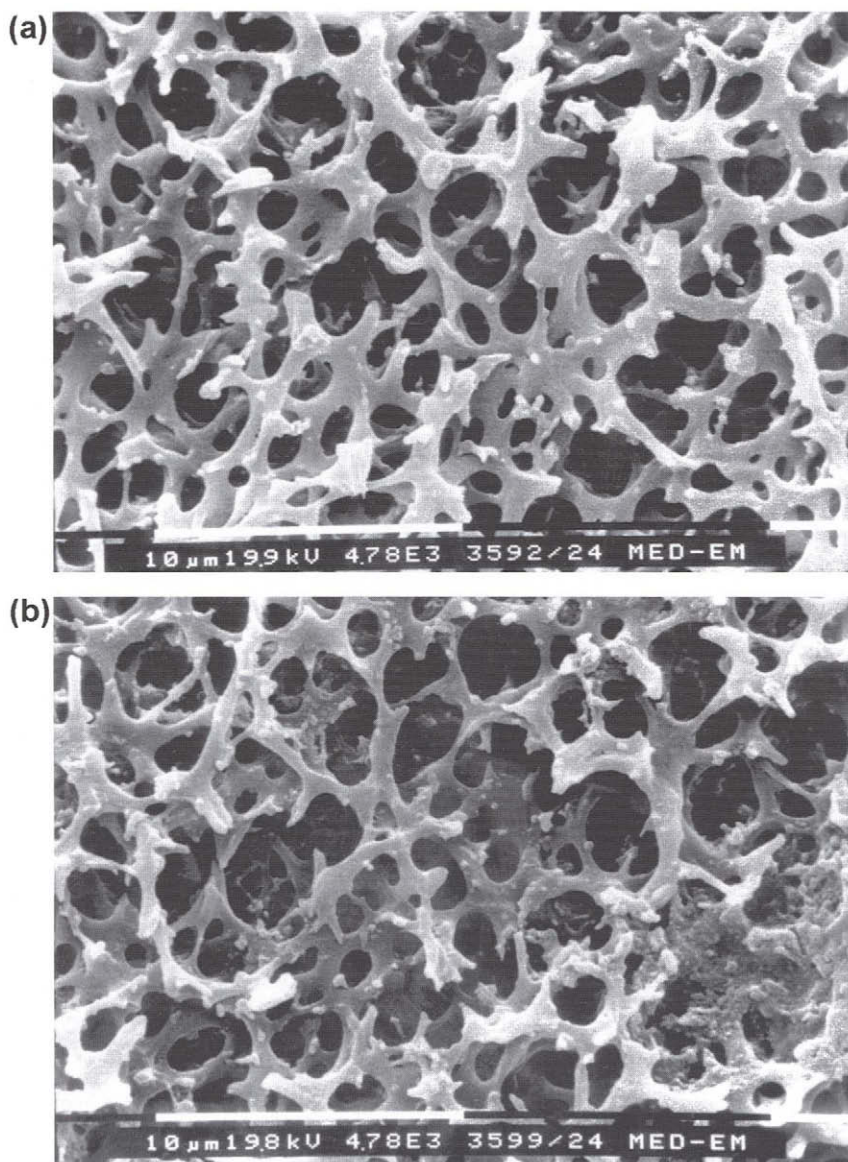
Fig. 9.5. Scanning electron micrographs of microfiltration membranes after cross-flow filtration of 40 mg/l humic acid containing pesticide samples: (a), fresh nylon membrane, and (b), after 16 h of use.

Contaminated effluents are, in most cases, highly complex and it is not an easy analytical task to make appropriate qualitative and quantitative determinations. The total characterization of effluents, to detect all compounds of particular interest, with discrimination against the interfering matrix is difficult by standard GC–MS methods. This is a result of problems in analyzing the more polar or non-volatile fraction of these organic compounds.

Castillo and Barceló have reviewed the ways in which the wide spectrum of endocrine toxic substances can be determined at low concentrations by means of CLC–MS [13]. It has been found that endocrine disrupters in humans may not appear until long after exposure. Organic pollutants reported to have reproductive and endocrine-disrupting effects include pentachlorophenol and penta- to nonyl-phenols [14].

There is a growing awareness of the area of non-ionic surfactants. Environmental bio-degradation will result in transformation products of alkylphenols and short chain alkyl-phenol ethoxylates which are more persistent than the parent compounds and may accumulate in food chains which have also been shown to elicit weak oestrogen activities [15]. The highly complex composition of these environmental samples puts a large demand on developments in analytical methodology and increasing pressure to improve legislations controlling environmental waste [16].

De Voogt et al. summarized well the determination of alkylphenols and short chain alkylphenol ethoxylates by CLC and GC–MS applied to waste waters and sewage sludge samples taken from industrial plants. They found predominantly oligomers containing one to three ethoxylate units [17]. Recently there has also started a program within the EU to develop European soil databases to be used as a tool for risk assessment and decision-making. This will allow for regional and local environmental risk assessments that would have indirect links to the water quality.

2-Hydroxymethylphenol, 3-hydroxymethylphenol, 4-hydroxymethylphenol, 2,4-di(hydroxymethyl)phenol, 2,6-di(hydroxymethyl)phenol and 2,4,6-di(hydroxymethyl)-phenol, are known to be contact sensitizers to humans, and are present in resins based on phenol and formaldehyde [18]. The cross-reaction patterns of these phenols which cause hypersensitivity in patients make it necessary to know their abundance, for diagnostic, therapeutic and preventive reasons. Nineteen substances were tested at equimolar concentrations and in serial dilutions. Reversed phase CLC separations were carried out to exclude contamination as the cause of the skin-patch test reactions. It was found in this study that probable cross-reacting substances were 2-cresol, 4-cresol, salicylaldehyde, 2,4–dimethylphenol, and 2,6-dimethylphenol [18].

Baranowska and Pieszko [19] developed methods using thin layer chromatography for CLC separations of methyl- and chloro- derivatives of phenols on bonded amino-, cyano-, and diol groups, using as binary mobile phases chloroform - propan-2-ol, 50 + 1.5 (v/v); chloroform - propan-2-ol, 50 + 4 (v/v); and *n*-hexane - propan-2-ol, 50 + 0.5 (v/v). Reversed-phase separations using ODS2 column material were used for stability determinations of the plastics additives, 2,4-dihydroxybenzophenone, 2,2′-dihydroxy-4-methoxybenzophenone, 2-hydroxy-4-*n*-octyloxybenzophenone, and 2-ethoxy- 2-ethyloxanilide [20]. The effect of the packing pressure on the performance of C-18 reversed-phase CLC columns was investigated systematically with Kromasil and Zorbax (10 μm spherical C-18), using chloroform as the slurry solvent and methanol as the pushing solvent [21].

Early evidence has shown that CLC columns packed with a given ODS phase give different performances, related to differences in their packing densities and external porosities, for benzyl alcohol, 3-methylphenol, 2,6-dimethylphenol, methyl benzoate, and β-phenylpropan-1-ol. This is of great importance in the design of separation systems to be applied to highly complex water samples. The current work permits a reasonable prediction of the band profiles recorded on one column by using isotherm data measured on

another column. Considering the difficulties encountered in volume determinations, normalization of isotherm data by the packing weight seems offer the most practical solution [21].

Tsuruta et al. developed a fluorometric method for the determination of phenol and 4-methylphenol in urine by precolumn high-performance liquid chromatography using 4-(*N*-phthalimidinyl)benzenesulfonyl chloride [22]. Precolumn derivatization was made where phenol and 4-methylphenol (*p*-cresol) were extracted with diisopropyl ether and derivatized with 4-(*N*-phthalimidinyl)benzenesulfonyl chlorideN-phthalimidinyl)benzenesulfonyl chloride",4> to give fluorescent sulfonyl esters. The labeling reactions were completed at 75°C for 10 min. The fluorescent derivatives were separated by reversed-phase separation and gradient elution using acetonitrile–water and detected by fluorescence. It was found that the mean concentrations of phenol and *p*-cresol in normal human urine were 67.3 and 167.9 nmol/mg creatinine, respectively. Multicomponent phenols were also determined selectively by fluorescence detection using partial-least-squares multivariate calibration [23]. Excitation fluorescence spectra were recorded between 210 and 285 nm, with an emission wavelength of 298 nm. The excitation emission spectra of these compounds are strongly overlapped, which precludes their direct determination without previous separation by conventional methodologies. The correlation was found by a validation applied to the determination of phenol, *o*-cresol, *m*-cresol and *p*-cresol in natural water and soil samples previously spiked with different amounts of each solute.

The efficiency of bioremediation, emphasizing the detoxification and destruction by micro-organisms of toxic substances in highly polluted industrial waste waters is measured efficient using reversed-phase CLC separation [24]. Besides phenol, a large variety of organic compounds was identified. The results show that from an initial phenol concentration of 987 ppm, slightly more than 50% was destroyed within 163 h. This suggested that the phenol was consumed by the micro-organisms as the sole carbon source.

Phenolic waste waters from a local oil-shale industry were analyzed by the luminescent bacterium *Photobacterium phosphoreum* [25]. The methodology could analyze activated sludge for its phenolic composition using CLC, and for its relative toxicity, and with Microtox™ and Biotox™ tests where the inhibition of natural luminescence of photobacteria is used as a toxicity endpoint. The most abundant components in ash-heap water were phenol (84 mg/l) and *p*-cresol (70 mg/l). Additionally, the toxicity of AHW towards two different activated sludges (acclimatized, and not acclimatized to phenolic wastewater) was evaluated using the decrease of adenosine triphosphate (ATP) content of the sludge as toxicity endpoint. New detection methodologies for the elucidation of 4-chlorophenol from the photolysis mechanism was made by a Fourier Transform electron paramagnetic resonance system developed by Martino et al. combined with liquid chromatography [26].

Water-soluble unbound phenolic compounds from leaves from the Mediterranean area was investigated and their concentrations assessed using reversed-phase CLC [27]. Ferulic acid, phloridzin, phloroglucinol, *p*-anisic acid, acetosyringone, sinapic acid, phenol, *p*-hydroxybenzoic acid, *p*-coumaric acid and cinnamic acid represent more than 95% of the mean relative concentration of phenolic compounds measured. Leachates from lignin-derived phenols in sediments from lignitic remains contain monomeric phenolic structures which can be regarded as specific bio-geotracers. They were found to contain ketonic, aldehydic and acidic forms of 4-hydroxybenzyl-, guaiacyl- or vanillyl-, syringyl-, and cinnamyl acids [28].

Vanbrujnsvoort et al. developed a method for the determination of chlorophenols by micellar electrokinetic chromatography (MEKC) coupled with electrochemical detection [29]. Nineteen chlorophenols and phenol were also separated utilizing a mathematical model to predict optimal separation conditions. Out of these, seventeen of the twenty compounds could be baseline-separated with AGES buffer at pH 6.1, with sodium dode-cylsulfate as the mobile phase. Detection was made by amperometric oxidation. A compensating pressure was applied to preserve the flat electro-osmotic flow profile during analysis: plate numbers up to 150,000 were obtained. PLRPS–SPE sample preparation was performed off-line with sample volumes of 100 ml: the method proved suitable for the analysis of river water samples [29]. Phenol and nitrophenols were determined by super-critical fluid chromatography (SFC) coupled to an SPE step with C 18, PLRP materials [30]. Separation was achieved in less than 6 min, using the SPE step to improve the sensitivity: the solid-phase extraction was used to reduce the limits of detection. Ion-pair mechanisms were utilized, with tetrabutylammonium bromide as the ion-pairing reagent in the extraction process to increase breakthrough volumes, mainly for phenol. The performance of the method was investigated with both tap- and river waters [30].

9.3.1 Mass spectrometry

Puig et al. [31] investigated on-line liquid–solid extraction sample-clean-up coupled to an analytical CLC-separation of river-water samples with nineteen priority phenols. Mass spectrometry detection was studied, comparing atmospheric pressure chemical ionization (APCI) and ion-spray (ISP) interfaces in the negative ionization (NI) mode. Sixteen out of the priority methyl-, nitro-, and chlorophenols were determined by LC–APCI-MS with high sensitivity. Parts-per-trillion levels of detection were found for 50–100 ml of river water, processed in full-scan and time-scheduled SIM modes. The same group also studied three different liquid chromatography–mass spectrometry interfacing techniques - thermo-spray (TSP), atmospheric pressure chemical ionization (APCI), and ion-spray (ISP), using the negative ion mode (NI) for the analysis of priority phenols [32]. The TSP interface resulted in $[M–H](-)$ or $[M+CH–3COO](-)$ as the main ions. APCI and ISP interfaces gave $[M–H](-)$ as the main ion with an optimal extraction voltage in the range 20–30 V. Some solutes (phenol, 4-methylphenol and 2,4-dimethylphenol) were only ionized by ISP by raising the organic modifier percentage to 100%. The instrumental detection limits obtained with ion-spray and the thermospray were in the same range, but were improved by one order of magnitude when the ACPI interface was used. A number of chloro-, nitro-, and methyl- substituted phenols have been determined in soil samples [33] using the same CLC–APCI–MS methodology as above.

Matsumoto et al. have studied the enhancement of the molecular ion peak generated from phenols and other halogenated benzenes by applying femtosecond (150 and 500 fs) laser pulses in conjunction with supersonic beam–multiphoton ionization MS [34]. The molecular ions are strongly enhanced when the pulse width of the ionization laser is shorter than the lifetimes of their excited states. Specifically, in the case of 2-chlorophenol, intersystem crossing was found more efficiently by stabilization of the triplet state by intramolecular hydrogen bonding. The results from this group indicate that this laser technique is useful in improving the ionization efficiency with molecules having short lifetimes.

9.3.2 Electrochemical detection

Electrochemical detection (ED) principles are often characterized by high sensitivity and offer good compatibility with CLC systems. Some limitations, such as the high operation potentials, appear for a large number of other electrochemically active compounds to be oxidized or reduced. This is generally the case in complex samples where the background signal originating from interfering compounds present in the matrix will give rise to misinterpretation in both qualitative and quantitative determinations. With proper sample preparation and liquid chromatographic separation columns this can, in many cases, be circumvented.

Different techniques, for example coulometric, can be used for the determination of phenols in water samples [35,36]. Furthermore Amperometric detection of substituted phenols [9,37] and pulsed amperometric detection principles are applicable. An advantage in sensitivity over UV detection was found for a number of phenols when amperometric detection was used [37]. The disadvantage found in this work was the oxidation of nitrophenols.

Another electrochemical approach has been reported where a combined amperometric and potentiometric measurement was applied in combination with micro-CLC for the determination of phenols [38]. This is, however, often associated with problems due to the co-oxidation of interfering substances and the high risk of polymerization reactions of the phenols which result in fouling of the electrode surface. Additionally, non-reversible electrochemical reactions occur at the electrode surface which can lead to poisoning and electrode fouling. This is seen as a time-dependent increase in the background current, as well as a reduction in sensitivity of the electrochemical detection. Attempts have been made by some groups to circumvent this. Pulsed amperometric operations can be made by stepping the potential in both oxidative and negative directions, thereby cleaning the surface continuously and regenerating a new, freshly exposed electrode area. Knutsson et al. have developed amperometric detections for the simultaneous determination of five chlorinated phenols with one to five chlorine atoms (4-chlorophenol, 2,5-dichlorophenol, 2,4,5-trichlorophenol, 2,3,5,6-tetrachlorophenol, and pentachlorophenol) reaching sensitivities below 0.1 μg/l [9].

Composite graphite–poly(tetrafluoroethylene) electrodes were developed as amperometric indicator electrodes in HPLC detection for the selective determination of phenol [39]. The CLC separation was transported to a wall-jet flow-cell configuration. Vanbrujnsvoort et al. used amperometric detection in MEKC with a graphite epoxy working electrode at a potential of 800 mV vs. Ag/AgCl. A palladium metal union was used to decouple the separation field from the electrochemical cell [40].

The limitations mentioned above, of electrode fouling when using electrochemical transduction can be circumvented by using biocatalysts. The introduction of a biological recognition element such as an enzyme makes it possible to use these proteins as mediators, allowing the electrochemical transduction to be operated at much lower potentials [41].

9.4 SOLID PHASE EXTRACTION METHODOLOGY

There is a striving towards exchanging the liquid–liquid extraction (LLE) methodology

and replacing it by SPE methodology, thereby circumventing the use of large volumes of toxic organic solvents. As a result, improved sample handling yields can be obtained without major losses of analytes. It will also be possible to fully automate the whole sample handling procedure, including sample delivery, and adsorption–description processes, as well as additional tedious manual experimental steps. By using robots, totally unattended operations, including data acquisition can be achieved. A large number of SPE sorbents has been used for various water samples, aiming for optimal adsorption–desorption properties resulting in high recoveries with additional selectivity [42]. In most cases, the physico-chemical affinity between the sorbent and the analyte lacks the selectivity required to achieve trace enrichment of the solute alone. Starting with large volumes of water will produce concentrated extracts that also contain a large number of matrix compounds that might present additional problems for CLC separation with conventional detectors. Here, the degree of detector selectivity will play a major role in obtaining proper quantitative results.

Highly cross-linked polymeric materials have also been applied commonly in the preconcentration of phenols and many other substituted aromatic compounds. The stabilities of the phenols are of great importance when it comes to sampling, sample storage, and finding storage times that can be allowed without degradation. This is also linked directly to the analytical capacity that environmental laboratories have today, despite the fact that automated analysis equipment is commonly used. Castillo et al. investigated various polymeric sorbents (Isolute ENV+, Lichrolut EN, and Porapak RDX) for the analysis of fourteen phenolic compounds included in the priority pollutants list of the US Environmental Protection Agency and in the European Union list [43]. These were: catechol, phenol, 4-nitrophenol, 4-methylphenol, 2,4-dinitrophenol, 2-nitrophenol, 2-chlorophenol, 4-chlorophenol, 3-chlorophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, and pentachlorophenol.

Recoveries ranged from 70 to 100% (except for catechol) on sample volumes of 700–1000 ml (5 μg/l, pH 2.5–3.0). The stability of the target compounds on Isolute ENV+ was evaluated by storing the sorbent material at −20°C, 4°C, and at room temperature for up to three months. Complete recovery was observed after storage at −20°C for two months. At room temperature, losses of up to 70% were observed for phenol, catechol, and the more volatile phenols. The group found that the stability of the phenolic compounds was dependent on the water matrix, the storage temperature, and on their physico chemical properties, such as vapor pressure and water solubility.

The polymeric sorbent Lichrolut EN was also used for the determination of three organophosphorus pesticides with phenolic-type structures (fenitrothion, ethyl parathion, methyl parathion) and pentachlorophenol in natural waters from Portugal [44].

All transformation products were found to be more stable than the parent compounds, except in groundwater conditions. Half-lives of 5 and 4 days were calculated for 4-nitrophenol and paraoxon ethyl, respectively, in estuarine and river water. Values of 3 and 4 days were obtained for 3-methyl-4-nitrophenol in estuarine and river water, respectively. This was applied to three different types of natural waters (river water, estuarine water and groundwater).

Puig et al. made a comparative study of the performance of four sorbents: PLRP-S, LiChrolut EN, Isolut ENV, and porous graphitic carbon (PGC), for on-line liquid–solid extraction (LSE) followed by CLC-separation of phenolic compounds in water [45]. The

highest capacities in terms of breakthrough volumes were obtained for the LiChrolut EN and Isolute ENV. Varying recoveries were found, at 55–105%, except for catechol and 2-amino-4-chlorophenol. Detection limits of 0.1 μg/l were reported in groundwater samples. The methodology developed was also validated by inter-laboratory exercises using groundwater samples distributed by Aquachek (WRC, Medmenhan, UK), containing phenols at levels ranging from 0.1 to 5 μg/l. Equilibrium between bound and free phenols in humic-containing waters was realized, which makes the quantification an important challenge. The LiChrolut EN sorbent was also compatible when coupled on-line to reversed-phase separation and high sensitivity electrochemical detection [46].

SPE is gaining much attention in the immunoaffinity-based 96-well plate assay techniques. Outside traditional stationary phases, several groups have specialized in immunoaffinity sorbents that have specific and selective binding of the analytes to the immobilized antibody in the column. Even on-line approaches have been presented which have excellent performance, but this area will not be described here. There are several reasons why this is so important for the ELISA type of immunoassays: (i), sample volumes are freely available, while assays generally make use of 5–200 μl volumes; (ii), sensitivities can, in most cases, not be reached by direct analysis; and (iii), matrix complexity may often ruin the affinity binding between antibody and tracer on one hand and antibody and antigen on the other. Humics represent such complex compounds containing interferents. Humic substances are, by definition, a group of compounds with very high variety in both molecular weight and structure composition. A number of SPE materials has been used for this purpose, e.g. PLRP-S sorbent, Hypersil green ENV, and Lichrolut EN [47,48]. Pentachlorophenol in certified waste waters, soil samples, and industrial effluents was determined using a commercial assay kit (RaPID magnetic particle-based ELISA (Ohmicron; Newtown, PA, USA) [47]. The cross-reactivity experiments in the ELISA revealed the affinity of tetrachlorophenols, enhanced in the presence of PCP, and discrepancies between the ELISA and LC results were observed when the concentration of 2,4,6-trichlorophenol was 1.5–2 fold higher than that of PCP. The results obtained for contaminated soils by ELISA and LC were in good agreement.

A selectivity–capacity comparison was made with eight sorbents packed in a precolumn coupled on-line with a liquid chromatograph with UV detection [48]. Eighteen priority phenolics listed in the US EPA were used as the reference solutes. The method was compared with an off-line methodology using Empore extraction disks and some 100 ml of water samples. Hydrophobic interaction by preconcentration on phenyl columns was developed for the study of radiation-induced decomposition of chlorophenols [49]. It was found that the efficiency depends substantially on the radiation dose used and the presence of specific scavengers in the irradiated samples. This is one of the first studies showing that the increase of radiation dose results in gradual elimination of chlorine atoms from the chlorophenol molecules.

Phenols also show binding properties to C-18 Empore extraction disks [50]. In this early work liquid chromatography was coupled on-line with rapid-scanning UV–VIS detection and post-column fluorescence detection for the isolation and trace enrichment of various pesticides such as carbamate transformation products of some phenolic structures - 3-hydroxy-7-phenol, 3-keto-carbofuranphenol, aldicarb sulfoxide, aldicarb sulfone, 3-hydroxycarbofuran, carbofuran and 3-ketocarbofuran. At levels of 0.2 and 5 μg, about 50–100 ml of drinking water sample was required. However, using the same on-line system

coupled with LC–post-column derivatization, fluorescence detection needed only 10 ml of water to achieve similar levels of determination for the carbamate insecticides. Chee et al. coupled this technique to a closed-vessel microwave extraction system [51]. The recoveries of eleven phenolic compounds spiked at 10 and 500 µg/l levels into water, using the optimum conditions, were all above 85% with relative standard deviations between 4.0 and 10.0%, except for phenol and 4 nitrophenol. Similar recoveries were found in industrial wastewater samples to those given by LLE and C-8 SPE cartridge techniques. More recently, graphite-based sorbents have been used for the enrichment of polar phenols. Highly apolar phases, such as various graphite materials, are useful for trace enrichment of polar phenols. Crescenzi et al. investigated the performances of two new graphitized carbon blacks (GCBs), Carbograph 4 and Carbograph 5, which were evaluated as SPE materials [52]. Compared to an older GCB, Carbograph 1, the two new GCBs exhibited far larger abilities to extract very polar compounds, and proved to be efficient at enriching both drinking water and humic-containing waters. Puig et al. reported that porous graphitic carbon (PGC) has good binding properties, especially for aminophenols [44].

A somewhat different approach was taken by Zhi et al. in which phenols were determined selectively in waters and soil leachates down to sub-µg/l levels, using a continuous flow spectrophotometric method [53]. In this method, the analytes were preconcentrated and separated from the sample matrix by on-line sorption onto a commercially available column packed with an ion-exchange stationary phase, Amberlite XAD 4, at pH 2.0 in a flow system. Aromatic amines that interfere in the detection using a 4-aminoantipyrine method were not retained, which allows the elimination of the CLC separation column in this system. Retained analytes were eluted by continuously pumping an alkaline aqueous stream at pH 13. The calibration graph was linear over the range 0.5–60 ng/ml and the detection limit was 0.2µg/l. The sample throughput was 8 samples per hour, with a preconcentration time of 5 min.

Puig and Barcelo have made a survey of current methodologies for the determination of phenolic compounds in various water types, comparing liquid–liquid extraction (LLE) and liquid–solid extraction (LSE) combined with liquid chromatography [54].

9.5 CLC-BIOSENSOR DETECTION

There is a considerable effort to use bioselective detection systems to reduce the cost of analysis, but also to substantially improve the selectivity as compared with classical techniques. The analytical problems encountered with conventional detection techniques has recently led the European Union (EU) to initiate a research program within the environmental program, devoted exclusively to the development of bioselective detection for environmental control. Recent progress in studies of detection methods for analytical flow systems based on biological recognition (biosensors based on enzymes or antibodies) in the author's laboratory are presented, focusing on the selective detection of representative analytes for environmental needs [41,55–75].

One of the key problems in analytical chemistry is selectivity, particularly at low analyte concentrations and in the presence of interfering substances. In most cases, conventional CLC detectors such as UV, refractive index, and electrochemical detectors

lack this necessary selectivity. This is where biospecific detection has proved to be of great potential to the analytical chemist.

The term biospecific detection can include any type of biochemical recognition reaction which can be transformed by an appropriate physical transducer to an electrical signal of some sort. These biochemical components can be of enzyme-, whole cell-, whole tissue-, nucleic acid-, receptor-, or antibody origin. The immobilized species can be of enzyme or antibody–antigen origin, where the substrate or antigen–antibody fits in a perfect lock and key configuration. In this section, enzyme-based detection systems for phenolic compounds will be presented.

Enzymes are flexible molecules with a large variety of molecular weights and complex conformations, which are generally linked to their catalytic activity. One enzyme can be obtained and purified from several biological sources, which results in preparations having different properties. They demand defined operation and storage conditions, which limit their activity and stability. They are water soluble and water, to a variable extent, is always required for their solubilization. They have very well-defined binding sites for the substrate (analyte). Many of them require the presence of cofactors which can be firmly attached to the protein (prosthetic group) or have to be added (coenzymes). They may be easily activated or inactivated by the presence of other compounds.

One limitation in interfacing enzyme-based detection to CLC systems relates to the use of organic modifiers in the mobile phase. Organic solvents such as methanol or acetonitrile are, in most cases, detrimental to the activity and stability of the enzyme. As a compromise that can be handled, a small amount of organic solvent is used in combination with a shorter separation column. Tyrosinase, laccase and peroxidase originating from various sources are by far the most commonly used enzymes for the analysis of phenolic compounds, for both CLC and FI-purposes. Additional biosensors using tyrosinase and a working electrode of a glassy carbon type, modified by co-immobilization of tyrosinase and a conducting poly(1-vinylimidazole)-based osmium polymer on its surface was developed by Adeyoju et al. [76].

CLC separation using binary mobile-phase mixtures of methanol and phosphate buffer, followed by the chemically and enzymatically modified electrode, was applied to *p*-amino- phenol, phenol, *p*-cresol, catechol and *p*-chlorophenol determinations. The biosensor exhibited a 100–200-fold improvement in sensitivity and detection limits when compared with a UV–VIS spectrophotometric detector.

High sensitivity improvements were shown by using short-chain hydrocarbons used as pasting liquids in carbon-paste tyrosinase amperometric biosensors [77]. The change in the carbon-paste binder also influences the selectivity of the tyrosinase electrode and the K-app. values. In flow-injection mode, detection limits of 6 nM catechol and a relative standard deviation of 2.5% ($n=30$) were obtained. A dual-enzyme electrode chromatographic detection, based on the use of different pasting liquids, provides unique characterization of the phenolic substrates. The merits of this strategy are illustrated in connection with a river-water sample. Such sensitivity enhancements are attributed to the extractive accumulation of the phenolic substrates. In another study, the suitability of organic media was investigated by kinetic studies of tyrosinase-based biosensors [78]. Covalently immobilized tyrosinase on microporous gold electrodes was developed as a flow-through biosensor–electrochemical detector in both CLC and flow injection analysis [79]. A very low operational potential of −50 mV (vs. Ag/AgCl) could be used, gaining detection
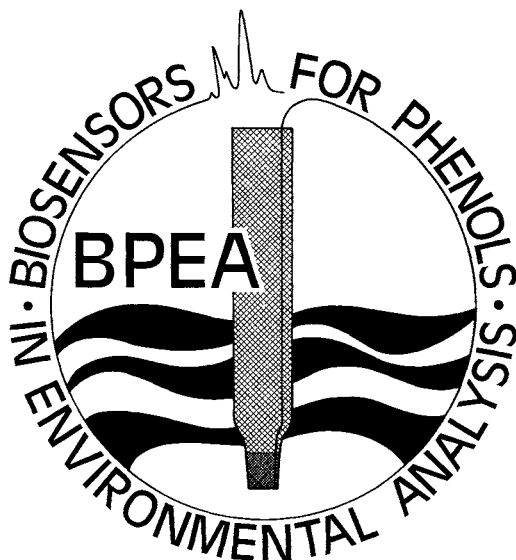
Fig. 9.6.

limits of 0.5 μM catechol and 3.4 μM phenol, respectively, with a sample throughput rate of 38 samples/h. The electrode is shown to be stable for up to 14 days' use.

9.5.1 Determination of phenols by integrated analytical flow systems

A large number of biosensor principles and configurations has been developed over a period of five years in a number of EEC and EU projects within our group, for both CLC and FI systems to determine phenols in environmental waters [41,55–75]. Fig. 9.6 illustrates the logo for one of these projects in this programme.

Tyrosinase-modified carbon paste and solid graphite electrodes have been used for screening analysis of some phenols in environmental waters in Spain and Sweden. Factors such as the mode of screening operation, analysis time, and sensitivity were optimized for each flow-configuration system. In our laboratory, a totally integrated on-line flow system incorporating sample clean-up with trace-enrichment, chromatographic separation, and biosensor detection, was developed for the screening for phenols in surface-water samples. The chromatographic system is depicted in Fig. 9.7. Preconcentration was necessary in order to detect the analytes in the lower ppb region. The selectivity and sensitivity in this system were obtained through the combination of the sample pre-treatment step using SPE and the biosensor detection based on the phenol-selective enzyme, tyrosinase. Optimal desorption kinetics in SPE usually require the use of rather large amounts of organic modifier for transfer of enriched analytes from the SPE column. This was found to limit the biosensor performance. High emphasis was put on interfacing conventional SPE techniques with biosensor technology so that both techniques are compatible and perform optimally. Initially, the large volume of acidified sample was loaded onto the SPE column. In a second step, the SPE column was washed with an aliquot of water prior to desorption. Desorption was made by the use of an acetonitrile–water plug. A heart-cut sampling was

made, collecting the main fraction into the loop of the switch valve. Next, the loop content was introduced into the small separation column where the organic front was sent to waste and the separated phenols to the biosensor. Desorption was performed in two consecutive steps for each sample. In the first desorption step, a smaller amount of organic modifier was used to desorb the most polar phenols such as catechol and phenol, and in a second desorption step those with more hydrophobic properties such as p-cresol and 4-chlorophenol. We also found that if the level of organic modifier is too high, the resolution between the analyte peak and the organic front in the separation step is lost. Of the two different biosensor configurations - a solid graphite electrode, and the carbon paste electrode - the highest sensitivity was obtained with the solid electrode. A number of water samples was analyzed, where seven different spiked surface-water samples from the Ebro Delta in Spain was obtained and screened with the system. Surface-water samples spiked with an unknown concentration of catechol were determined using this approach. In this way, a number of samples could be screened for the presence of phenolics at 1–25 μg/l levels in humic-containing surface waters. A corresponding representative separation is shown in Fig. 9.8, where the arrows indicate the two different desorption steps. We also found that, in comparison with UV detection, detectability becomes a major problem in the analysis of surface-water samples from the Ebro delta. It was evident that the enzyme is superior to UV detection in its selectivity towards phenol.

Toxcity aspects of hazardous phenols was also studied by the same type of biosensor (carbon plate) integrated with SLM sampling from plasma SPE displacement in the alkaline mode was linked to CLC and the biosensor, monitoring phenolic levels [96].

CLC was also combined with ELISA in an off-line mode by Krämer et al. [80]. They separated five 4-nitrophenols by C-18 modified CLC including acetonitrile (16%) and methanol (16%) in the mobile phase, followed by fractionation. The organic solvents in
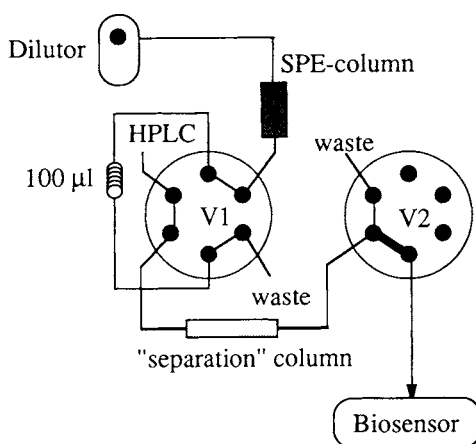
Fig. 9.7. An automated coupled column liquid chromatographic system integrated with an amperometric tyrosinase modified electrode. A 10 ml mixture of 10 ppb of each analyte using a short SPE column (PLRP-S, Polymers Lab., 20 μm, 2 × 10 mm) and a short analytical column (LiChrosorb, Merck, 10 μm, 2 × 30 mm) were used in the analytical system.

the fractions were evaporated and redissolved in appropriate ELISA-compatible aqueous eluents.

9.6 BIOSENSOR DEVELOPMENTS

In our group we have focused on fundamental studies of the performance of enzymes in close proximity to the electrochemical transducer where the enzymatically formed product is electrochemically oxidized or reduced. The basic understanding gives insights into the possibilities of using different biosensor concepts and an opportunity to target on the determination of one or a larger number of solutes. This basic understanding is of mandatory importance in developing the biorecognition principles used as detection units in chromatography and flow injection analysis (FI).

Different amperometric techniques are relevant to investigations of the fundamental aspects of an enzyme-modified electrode. Aspects such as the rate limiting steps, the electron transfer, and the amplification factor are basic to the optimizing of an enzyme electrode. Such investigations form the basis for improving the stability and sensitivity in a controlled manner. Typical techniques used for this purpose are cyclic voltammetry (CV), the rotating disk electrode (RDE), and chrono-amperometry (CA). Some of the various techniques are discussed below.

The biosensor consists of two parts – one biochemical component and one physical transducer – in close contact with each other. Exposure of the substrate (analyte) to the biological element results in a measurable electrical signal which is proportional to the concentration of the analyte. The main principles that have been developed for tyrosinase-, laccase-, and peroxidase-based biosensors aimed at the determination of phenolic compounds in environmental waters are presented below.

It is possible to design both highly analyte-specific biosensors, or more general group-specific biosensors. Solute-specific enzyme-based biosensors can be constructed by choosing an enzyme which catalyses the conversion of a specific solute structure, resulting in a narrow substrate selectivity. In a group-specific biosensor an enzyme is chosen which catalyses a whole group of related analytes with similar molecular structure, leading to a broader substrate selectivity. These two types of biosensors will enable the measurement of only a restricted number of phenols, or a larger number of phenolic compounds. Both types of biosensors have been developed on the basis of enzymes such as tyrosinase, laccase, and peroxidase, using different electrode materials, flow systems, and sample pre-treatment techniques [41,63,81,82]. In Table 9.1 we illustrate properties of the commonly applied enzymes, tyrosinase, laccase and peroxidase [106–108].

9.6.1 Tyrosinase modified biosensors

Despite the fact that phenol oxidases (tyrosinases, laccases) and peroxidases have different enzymatic mechanisms their actions in amperometric biosensors for the detection of phenolic compounds have similar reaction sequences, as shown in Figs. 9.9 and 9.10.

Enzyme molecules at the surface of the electrode are oxidized by oxygen (for phenol oxidases) or hydrogen peroxide (for peroxidase) followed by their re-reduction by phenolic compounds. During the latter reaction the phenolics are mainly converted into quinones and/or free radical products. These products are usually electrochemically active and can

TABLE 9.1

Properties of the commonly applied enzymes, tyrosinase, laccase and peroxidase

Properties	Tyrosinase	Laccase	Peroxidase
Source	Mushroom	Rhus vernicifera	Horseradish
Domains	Four	Four	Two
MW (kDa)	120–130	110–140	42
Electron-donating Groups	Four copper atoms per molecule	Four copper atoms per molecule	Ferriprotoporphyrin IX
Reference	[106]	[107]	[108]

be reduced at the surface of the electrode at more negative potentials than 0 V vs. the saturated calomel electrode (SCE). The reduction current is proportional to the concentration of phenolic compounds in the solution. Detection of oxygen or hydrogen peroxide
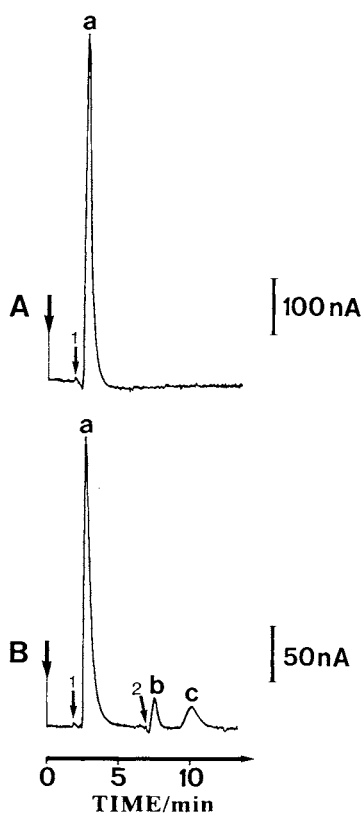
Fig. 9.8. The corresponding chromatogram is shown. One-step desorption (arrow 1) and two-step desorption (arrow 2) for a solid graphite electrode. In Fig. 9.2a, desorption of catechol and phenol simultaneously, and Fig. 9.2b, desorption of p-cresol. Conditions: eluent, a mixture of 90% 50 mM phosphate buffer, pH 6.0, and 10% of acetonitrile; flow rate 0.7 ml/min; the desorption mixture consisted of 30% acetonitrile and 70% water: applied potential −50 mV vs. SCE. Chromatographic separation with the integrated system described in Fig. 9.7.

$$Monophenol \xrightarrow[O_2]{\overset{Tyrosinase}{Step\ 1}} o\text{-}Diphenol \xrightarrow[O_2\ H_2O]{\overset{Tyrosinase}{Step\ 2}} o\text{-}Quinone$$
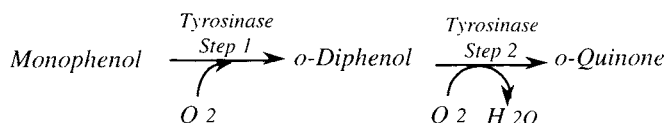
Fig. 9.9. The two enzyme reaction sequences for phenolic compounds.

consumption is another possibility for monitoring the concentration of phenolic compounds when using phenol oxidases (tyrosinase, laccase) or peroxidase. The advantages of an amperometric biosensor based on reduction of the enzymatic products are: (i), simplicity; (ii), protection of the electrode from the accumulation of polymeric products on the surface, which is usually observed during direct electrochemical or enzymatic oxidation of phenolics; (iii), amplification of the response as a consequence of the electrochemical re-reduction of quinones and phenoxy radicals to the initial phenolic compounds; and, (iv), the performance of electrodes in the optimal potential range for electrochemical measurements. Table 9.2 shows the comparison of published tyrosinase-modified electrodes for the detection of various mono- and di-phenols in aqueous solution.

One of the main points to consider in the development of amperometric biosensors is the possibility of operating within the optimal potential range, i.e. between -0.2 and 0 V vs. SCE [63]. In this region the background current switches signs, leading to low noise and background currents, and thereby enabling detection of low analyte concentrations. Additionally, the risk of interfering electrochemical reactions occurring in real applications is greatly reduced within this potential window. Environmental water samples contain varying amounts of electrochemically active matrix compounds such as humic substances, and thus special care should be taken when using electrodes operating at voltages outside the optimal potential region.

During the last few years a number of different electrode configurations with different electrochemical transducing materials and different enzymes have been developed. The choice of an analytical flow system will be determined by: (i), the complexity of the sample; (ii), the number of phenolic compounds one wishes to determine; and (iii), the number of samples to be determined. If a restricted number of phenols is of interest, biosensors with enzymes having narrow substrate specificity should be chosen. Only a few substrate phenols will yield a response for efficient and rapid catalysis. The other type of biosensor, with enzymes having broad substrate specificity will still be able to determine, with high selectivity, a larger number of solutes, e.g. 5–15 phenolic compounds with the help of trace-enrichment and the partial separation of analytes possible in CLC. If one wishes to have both qualitative and quantitative data, a CLC system is needed for the separation of each analyte. The analytical information needed therefore governs the design and type of biosensor system.

Tyrosinase is a highly active enzyme with favorable properties for biosensor use. A number of mechanisms have been proposed to explain these characteristics of tyrosinase. Rodríguez-Lopez et al. proposed a kinetic model of the action of tyrosinase, including both the phenolase and catecholase activity [83]. This was also presented in an additional work [84]. The mechanism considers the three different states of the enzyme, and that the enzyme has one active site. The enzymatic cycle of tyrosinase when o-diphenol and monophenol are used as the substrates is represented by the simplified reaction scheme shown in Fig. 9.10.

TABLE 9.2

Comparison of published tyrosinase-modified electrodes for the detection of various mono- and di-phenols in aqueous solution

Electrode	Enzyme immobilization procedure	Analysis mode	E appl/(mV)	Analyte	Sensitivity (AM/cm^2)	LDL[a] (nm)	Reference
Mediated reduction							
Carbon paste	Enzyme mixed with air-dried hexacyanoferrate and graphite powder	Flow injection	−0.2 V vs. Ag/AgCl	Phenol	0.02	150	[97]
Carbon paste	Enyme mixed with polymer-immobilized hexacyanoferrate(II) mediator with the graphite powder	Flow injection	−0.2 V vs. Ag/AgCl	Phenol	–	191	[98]
Solid graphite	Enzyme covalently immobilized on the surface of chemically modified electrode (TCNQ)	Steady-state	0.13 V vs. Ag/AgCl	Phenol	1.0	230	[99]
				Catechol	3.4		
				p-Cresol	1.0		
Direct reduction of o-quinone							
Glassy carbon	Tyrosinase electropolymerized in polyrole layer	Steady-state	−0.2 V vs. SCE	Catechol	1.5	2.0	[100]
				Phenol	0.4	5.0	
				p-Cresol	0.3	5.0	
				Dopamine	0.06	50	
				L-Dopa	0.04	800	
				L-Tyrosing	0.005	800	
Solid graphite	Covalently crosslinked tyrosinase on the carbodumide-activated graphite	Flow injection	−0.05 V vs. SCE	Catechol	2.2	2.3	[55]

Carbon paste	Enzyme adsorbed on the surface of the electrode	Steady-state	−100 mV vs. Ag/AgCl	Catechol	1.8	10	[101]
				Phenol	1.6		
				p-Cresol	0.91		
Glassy carbon	Enzyme mixed with polyvinyl alcohol / polyhydroxyl cellulose gel forming a freezing immobilization technique	Steady-state	−0.2 V vs. Ag/AgCl	Catechol	0.69	20	[102]
				p-Cresol	0.31	20	
				Phenol	0.18	100	
				Dopamine	0.06	100	
Graphite/epoxy resin composite	Lyophilized erizyme powder mixed with composite	Flow injection	−0.1 V vs. Ag/AgCl	Catechol	0.40	40	[5]
				Phenol	−	1000	
Graphite/Teflon composite	Lyophilized enzyme powder mixed with composite	Flow injection	−0.05 V vs. SCE	Catechol	0.01	200	[32]
Carbon paste	5% Ruthenium on carbon mixed with mineral oil, octadecylamine and enzyme	Steady-state	− 0.01 V vs. Ag/AgCl	Catechol	3.1	800	[103]
				3-Chloro-phenol	−	2000	
				Phenol	−	1000	
Carbon paste	Enzyme powder admixed during paste preparation	Flow injection	− 0.05 V vs. SCE	Catchol	0.82	600	[64]
				Phenol	0.20	420	
				p-Cresol	1.5	2050	
Carbon paste	Enzyme powder mixed with melted paraffin wax and activated graphite powder	Flow injection	− 0.2 V vs. SCE	Dopamine	−	50	[104]
Carbon paste	Mixture of graphite powder. tissue from egg-plant and mineral oil	Steady-state	− 0.2 V vs. SCE	Catechol	0.1	1000	[65]

[a] LDL = lower detection limit.

$$E_{met}(2Cu^{2+})Ph \;\overset{1.}{\rightleftharpoons}\; Ph + E_{met}(2Cu^{2+}) + Cat \;\rightleftharpoons\; E_{met}(2Cu^{2+})Cat$$

$$Q$$

$$\underset{2.}{}$$

$$E_{red}(2Cu^{+})$$
$$+$$
$$O_2$$

$$5. \qquad\qquad\qquad 4.$$

$$3.$$

$$E_{oxy}(2Cu^{2+}...O_2)Cat \;\rightleftharpoons\; Cat + E_{oxy}(2Cu^{2+}...O_2) + Ph \;\rightleftharpoons\; E_{oxy}(2Cu^{2+}...O_2)Ph$$
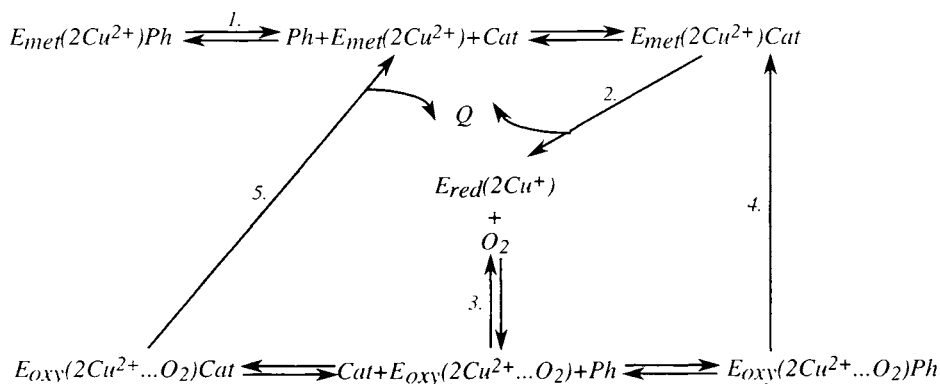
Fig. 9.10. The enzymatic mechanism of tyrosinase according to Rodríguez-López et al. [83].

As can be seen in this reaction cycle, the enzyme is mostly in the resting form. o-Diphenol removes the enzyme from the dead-end complex (step 1) and is enzymatically converted into o-quinone (step 2), while the enzyme is being reduced. The reduced form of the enzyme reacts with oxygen (step 3) and the oxidized form can either react with o-diphenol or monophenol. Monophenol is hydroxylated into o-diphenol (step 4) and o-diphenol into o-quinone (step 5).

Table 9.2 shows a comparison of published tyrosinase-modified electrodes for the detection of various mono- and di-phenols in aqueous solution [55,57,64,66,97–105].

The source of the enzyme will result in large differences in the selectivity of the biosensor. We investigated [69] the selectivity profile of carbon paste electrodes which were modified with tyrosinase originating from six different sources such as from different companies, different preparations, and origin. Nine phenolic compounds were included in this study. A variation of the selectivity pattern was seen between the electrodes which were modified with tyrosinase which originated from different sources [69].

Chemical modifications of electro-catalyzing mediators are used in order to shuttle the electrons from the enzyme to the electrode surface. Early work by Degani and Heller has shown the high potential of using osmium derivatives for a number of enzyme systems for this purpose [85].

We used a phenanthrolinedione as a redox mediator for the preparation of tyrosinase-modified electrode[69]. A higher signal for catechol was observed in the presence of the mediator compared with the direct reduction of o-quinones. This may result from a faster electron reduction for the mediated transport which, in turn, gives rise to a faster recycling, leading to a higher sensitivity. A hundred-fold reduction in the detection limit was observed for a tyrosinase-modified carbon-paste electrode modified with an osmium mediator (4,4'-dimethyl-2,2'-bipyridine)-2-(1,10-phenanthroline-5,6-dione) compared with an unmediated electrode [86].

9.6.2 Peroxidase-modified biosensors

Peroxidases exhibit relatively low specificity to electron donors and can be used for the determination of phenols [82]. Phenoxy radicals formed during the enzymatic oxidation of phenolic compounds in the presence of hydrogen peroxide are reduced electrochemically

and the reduction current is proportional to their concentration in the solution. From the hydrodynamic voltammograms, calibration curves, and performance stability it was concluded that the reduction of phenoxy radicals is more efficient at solid graphite electrodes than with the carbon-paste-based sensor. The potentials at which electrochemical reduction of phenoxy radicals appears depend on the electron donating properties of the substituent in the phenol molecule. Horseradish peroxidase (HRP) is relatively cheap and is commercially available in different purities. This haeme-containing glycosylated enzyme of about 44 kDa is a highly stable, efficient, and powerful catalyst. However, it requires the addition of hydrogen peroxide to the solution as the oxidizing agent. The catalytic cycle of peroxidase consists of the following reaction sequence, presented in Eqs. (2)–(5). The cycle starts from a two-electron oxidation of the ferrihaeme in the active site by hydrogen peroxide. This reaction results in the formation of an enzyme intermediate, compound I. The following two one-electron-reduction steps return peroxidase to the native resting state (Fe^{3+}). The phenolics being oxidized to free radicals will decay to non-radical products through polymerization or dismutation reactions, and thus a rapid electrochemical reduction of radicals is very important for obtaining sensitive and stable sensors based on peroxidases, and probably also on phenol oxidases.

The mechanism of peroxidase action

$$HRP(Fe_{III}) + H_2O_2 \rightarrow HRP - 1(Fe_V) + H_2O \tag{2}$$

$$HRP - 1(Fe_V) + AH_2 \rightarrow HRP - 2(Fe_{IV}) + \cdot AH \tag{3}$$

$$HRP - 2(Fe_{IV}) + AH_2 \xrightarrow{k_3} HRP(Fe_{III}) + \cdot AH + H_2O \tag{4}$$
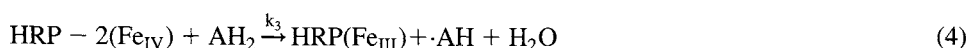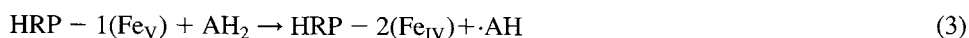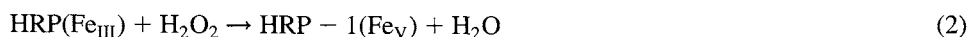
The native form of the enzyme reacts with the substrate hydrogen peroxide while the prosthetic group, protoporphyrin is oxidized into an unstable intermediate compound, HRP-1, whereby hydrogen peroxide is reduced into water. The two-electron re-reduction of the enzyme to its resting state is accomplished by a reducing substrate: AH_2 are various phenolic compounds such as p-nitrophenol, p-aminophenol, p-chlorophenol and p-cresol, as examples of reducing substrates. A free radical, $AH°$ is hereby produced and the enzyme forms a second intermediate compound, HRP-2 which reacts with another reducing substrate to finally form the native state of the enzyme. The last step is the rate-limiting step of the whole process. The free radicals formed through the last two reactions can react directly with molecular oxygen to give peroxy radicals which are converted into the corresponding hydrogen peroxide. These molecules can replace the hydrogen peroxide in the reaction.

It is known that in the presence of high concentrations of hydrogen peroxide a deactivation of horseradish peroxidase may occur, caused by the transformation of the prosthetic group into a higher oxidation level, compound-III (HRP-3).

$$HRP(Fe_{III}) \rightarrow HRP(Fe_{II}) \xrightarrow{O_2} HRP - 3 \tag{5}$$

Peroxidases isolated from different sources catalyse the same reaction but differ in their physicochemical properties and selectivity toward the various electron donors [73].

The mechanism of a HRP biosensor was studied for a number of phenolic compounds; phenol, catechol, resorcinol, p-cresol, 4-chlorophenol, 2,4-dichlorophenol, 4-chloro-3-

methylphenol, vanillin, and 2-amino-4-chlorophenol. The highest sensitivity was obtained for 2-amino-4-chlorophenol (85 nA/cm^2 μM) in the presence of H_2O_2 (10 μM in the solution). To our knowledge, this is so far the highest signal response ever reported. It was also found that the electrode was most stable when the buffer solution contained 5% of methanol.

Based on rotating-disk electrode experiments, it was concluded that phenoxy radicals produced in the peroxidase enzymatic cycle are effectively reduced electrochemically into their initial phenolic compounds at -0.05 V vs. SCE. The reduction currents start to appear at potentials more negative than the half-wave potentials, $E_{1/2}$, of the phenoxy radical–phenolic compound. Chloroperoxidase-modified graphite electrodes [62] exhibited responses for p-cresol similar to HRP-modified electrodes, confirming that the mechanisms of action of chloroperoxidase-modified electrodes are the same, or similar, at pH values around 7. However, in more acidic solutions a dependence of the response on the concentration of chloride ions was observed, owing to the pronounced ability of chloroperoxidase to halogenate phenolic compounds.

9.6.3 Biosensor configurations

There is a number of possible ways to immobilize biological catalysts in close proximity to an electrochemical transducer. Amperometric electrodes for determination of phenolic compounds have been constructed on the basis of: (i), surface-modified solid graphite electrodes where the enzyme is physically adsorbed, covalently immobilized, or membrane-retained, and (ii), bulk-modified composite electrodes, where graphite particles are enzyme-modified in similar procedures to those used in the preparation of surface-modified electrodes, followed by their mixing with oils, epoxy resins or Teflon particles.

The first enzyme-modified carbon-paste electrode was reported in 1988 [87]. Since
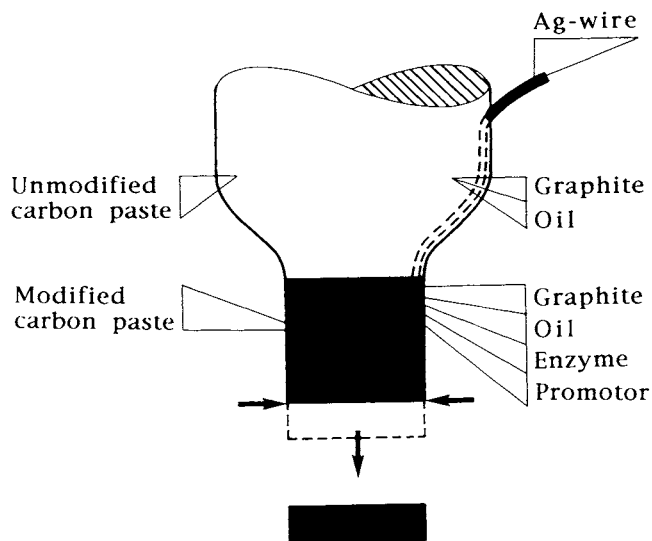
Fig. 9.11. Carbon-paste configuration of biosensor with the ability to generate a new fresh surface area by cutting off the top of the used layer.

then, the development of carbon-paste electrodes has seen steady progress. This sensor configuration offers a simple preparation at a low cost, and it is possible to modify the enzyme-modified bulk with additives and stabilizers. Additionally, when the enzyme layer is exhausted it can be replaced by simply repolishing the surface on a glass surface or a paper as shown in Fig. 9.11. Additives have been utilized for the modification of tyrosinase-modified carbon-paste electrodes, in order to improve the electron transfer and stabilize the enzyme [59]. Zeolites have also been introduced into carbon-paste electrodes in order to improve the reproducibility between batch modifications [70]. Epoxy–graphite [57] and Teflon–graphite [66] have, however, been shown to have great potential for obtaining good operational stability, even in the presence of organic modifiers.

9.7 IMMUNO-BASED BIO-RECOGNITION FOR THE ANALYSIS OF OTHER PESTICIDES

The problems involved in fast, sensitive and selective analysis of large numbers of samples require the development of new, highly efficient, automated, and more cost-effective screening techniques, which are ultimately aimed at unattended on-site screening - e.g. to give alerts of a sudden increase of environmental contaminants. Immunoassay techniques are potential candidates for fulfilling these requirements, owing to the high inherent selectivity of antibodies, the possible high sensitivity of such systems, and the fact that antibodies can be raised against virtually any compound. Most commonly, immunoassays are performed in the batchwise mode, but because of to the cost of automation and the lack of discrimination between cross-reacting compounds, new immunodetection principles are under constant development.

For the above reasons we have developed immuno-recognition systems with high sample-throughput properties using flow immunoassays which use restricted-access columns for the separation of the bound and free label for atrazine and its transformation products [74]. The transformation mechanisms of the parent compound are shown in Fig. 9.12. The immuno-flow system is shown in Fig. 9.13. We have demonstrated recent success in being able to perform flow-immunoassays on-line. Fluorescence-labeled antigens (triazines) have been used in a competitive assay format where samples containing triazines have been incubated for 2–5 min with labeled antigen and the corresponding antibody. A stoichiometric amount of triazines present in the sample will compete with the labeled atrazine forming the immuno complex, resulting in linear relationships between triazine analytes and the fluorescence signal response.

The method is based on the off-line incubation of the analyte (Ag), a fluorescein-labeled tracer (Ag*) and the corresponding polyclonal or monoclonal antibody (Ab). The separation of free (Ag*) and bound tracer (Ab–Ag*), is performed in a flow-immunoassay system, which incorporates a small column containing an alkyl-diol–silica restricted access (RA) material, previously described for a non-competitive assay by Oosterkamp et al. [88]. Similar flow-immuno systems have been published recently [89].

The RA-phase traps the free unbound tracer (Ag*) in its hydrophobic (C_{18}) inner cavity, but excludes the large Ab–Ag* complex, which is passed on and measured by a fluorescence detector. This type of system can, in principle, be applied to all small analyte molecules (<3000 Da) if the corresponding antibodies are available and is exemplified
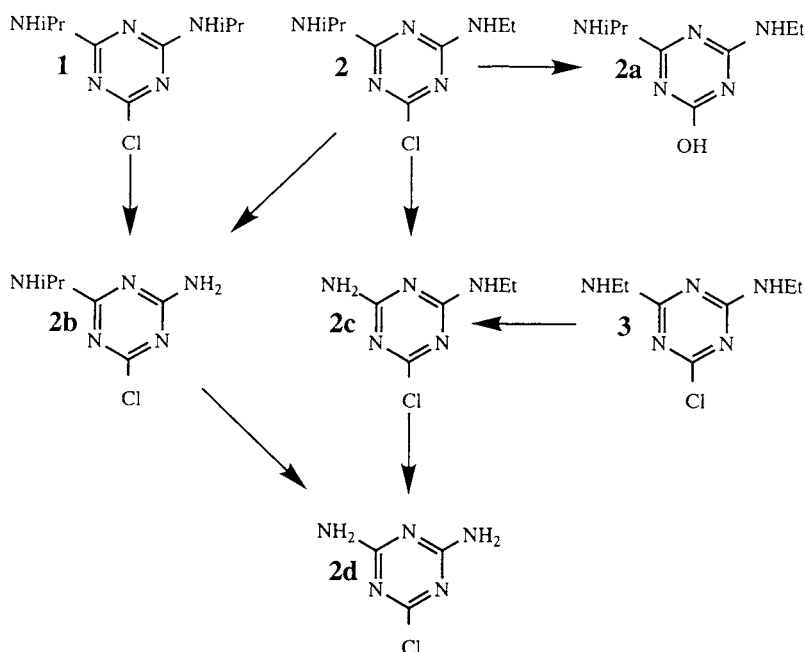
Fig. 9.12. Transformation scheme including some of the metabolites obtained from biological and chemical degradation of parent triazine compound: 1, propazine; 2, atrazine; and 3, simazine. The metabolites correspond to: 2a, hydroxyatrazine; 2b, desethylatrazine; 2c, desisopropylatrazine; 2d, didealkylated atrazine.

here for the screening of atrazine and its metabolites in a number of different environmental (surface water, waste water) and biological samples (human blood and urine).

The immunoassay principle is based on the fact that the analysis in the flow immunoassay follows a competitive immunoassay format where the actual detection is based on the fluorescence intensity of the antibody-bound tracer fraction (Ag*–Ab) according to the principle that the sample (Ag) is mixed and incubated off-line with optimized amounts of fluorescein-labeled tracer (Ag*) and antibody (Ab) until equilibrium conditions are attained.
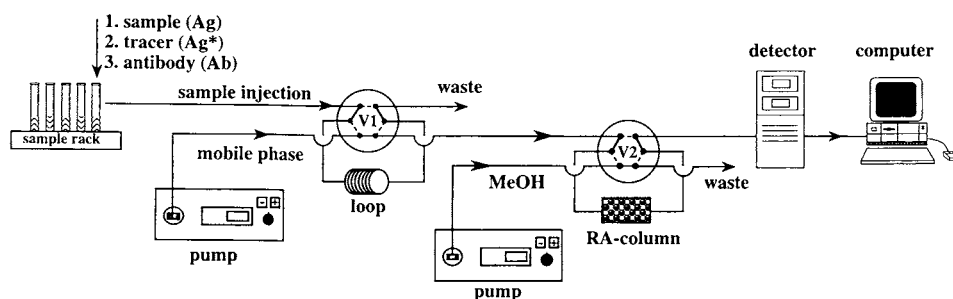
Fig. 9.13. Schematic overview of the flow immunoassay system.

The competitive reactions involved in the immuno-detection systems using fluorescent labeled antigens are

$$[Ag] + [Ag^*] + [Ab] \rightleftharpoons [Ag - Ab] + [Ag^* - Ab] \tag{6}$$
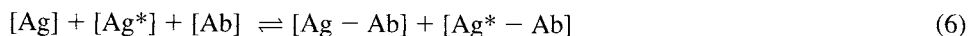
Separation of the free labeled antigen and the labeled antigen–antibody complex is achieved by using a restricted-access column on-line in the flow system according to the following reactions

$$Si - C18 + Ag^* + Ag + Ag^* - Ab \rightarrow Si - C18 - Ag^* + Si - C18 - Ag + Ag^* - Ab \tag{7}$$
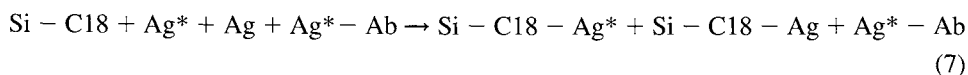
The reaction mixture is then injected into the flow system, where the unbound Ag and Ag* are retained by reversed-phase partitioning in the pores of the RA column material. The modified hydrophilic outer surface prevents unspecified binding of proteins, and a molecular weight cut-off of approximately 15 kDa prevents proteins from entering the hydrophobic inner surface. The immuno complexes Ag–Ab and Ag*–Ab are thus, because of their size, excluded from the pores of the RA material and pass unretained through to the fluorescence detector where the Ag*–Ab complex results in a signal peak that is stoichiometrically related to the Ag-concentration. The reaction principle in the RA-column is shown in Fig. 9.14. A low amount of target analyte in the sample gives a large fluorescence signal, resulting in a calibration plot with a negative slope.

The selectivity of an immunoassay given by the immunoreagent specificities, can be chosen according to the particular purpose of an assay. In cases where the metabolites and transformation products are of special interest for toxicity, biological activity, or other reasons. A broad specificity of the antibody is desirable. If, on the other hand, the determination of a specific analyte is of interest, antibodies with narrow specificity are to be preferred.
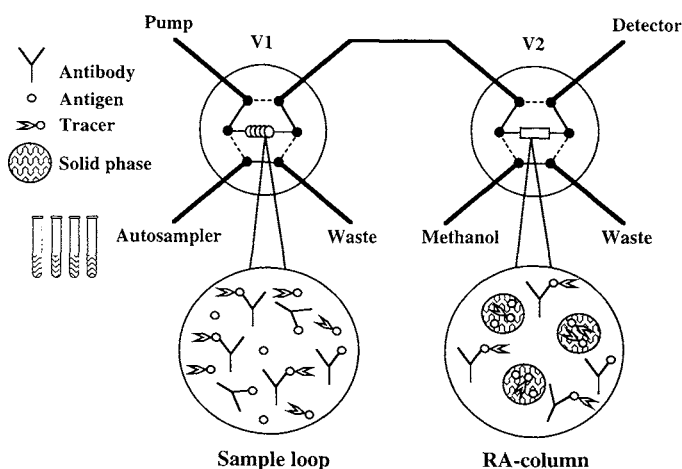
Fig. 9.14. Illustration of the immunoreactions taking place during incubation and in the separation step in the RA column. In the sample loop, all the reagents are present and at equilibrium. In the RA column, only the immunocomplexes are passed through, while the unbound antigens are hydrophobically trapped on the reversed phase.

The cross-reactivity of three of the antibodies in our study (Ab I, II and III) for seven different *s*-triazines were evaluated using fluorescence polarization [75] where the sensitivity for atrazine was set to 100%. We also found that Ab I has 20 and 33% cross-reactivity for only hydroxyatrazine and propazine, and can thus be considered to be a rather atrazine-specific antibody. Ab II and III, however, show significant cross-reactivity for several of the other *s*-triazines, and in the case of propazine even higher sensitivity than for atrazine. Thus, Ab II and III can be considered to be more group-specific than Ab I. The flow immunoassay was therefore optimized with the more atrazine-specific antibody, Ab I, for the screening of atrazine.

The displacement and association assay principles were optimized and, as already stated, the most sensitive competitive assay is obtained when the lowest possible amount of tracer is used. However, if the antibody affinities for the tracer and analyte differ considerably, the order of mixing the reagents can drastically change the sensitivity of the assay. A significant difference in sensitivity has previously been observed using fluorescence polarization immunoassay [1]. Three mixing orders are possible, giving different assay principles. The first is the association mode, in which pre-incubation of Ag with Ag* and then addition of Ab will give the Ab equal possibilities to bind either Ag or Ag*. The assay's sensitivity will be determined by the different affinities for the analyte and the tracer. In the second, the Ag-displacement mode, pre-incubation of Ag with Ab forms the Ab–Ag complex, and then the tracer Ag* is added. This assay will be less sensitive if the affinity for the tracer is lower than the affinity for the Ag, since the tendency to shift the equilibrium from Ab–Ag to Ab–Ag* is low. The third choice is the Ag*-displacement mode, in which pre-incubation of Ag* with Ab forms the Ab–Ag* complex and then the analyte Ag is added. For the same reasons as above, this assay format will be less sensitive if the affinity for the Ag is lower than for the tracer Ag*. One advantage of this last method is that a single reagent mixture, containing tracer and antibody (Ag*–Ab) can be used. The stability on storage of such mixtures is usually much better than of the two solutions separately.

The high sample throughput makes the assay very suitable for multiple injections of each sample, resulting in better confidence limits and more reliable data for applications within the biomedical and environmental areas. For example, during one working day, typically 400 injections were made on 90 samples (4–6 injections per sample). Within-sample and between-sample standard deviations were calculated using the (LSD) one-way anova for a 1 ng/ml atrazine sample. Six replicate measurements were run on eight separately prepared samples. The relative standard deviations (RSD) within- and between-samples were 1.9% (degrees of freedom, df=40) and 4.2% (df=7), respectively. The sample throughput was 80 samples per hour and the detection limits were 1.4 nM (300 pg/ml) for atrazine and 2.3 nM (500 pg/ml) for the sum of triazines. Water samples from Russia, with unknown concentrations of atrazine, and creek samples from Sweden spiked at various concentrations of atrazine, were screened in the developed flow immunoassay.

In order to be able to study the toxicity aspects of occupational exposure of atrazine, spiked human urine (see Fig. 9.15) and human blood and urine samples at the 0, 3.3 and 10 ng/ml levels, were processed in the same manner as the water samples above. We found that the urine samples could be analyzed successfully, while the properties of the plasma samples resulted in peaks that were significantly higher than the normal AbAg* peaks owing to the high protein content in plasma, with inherent native fluorescing with 0 ng/ml
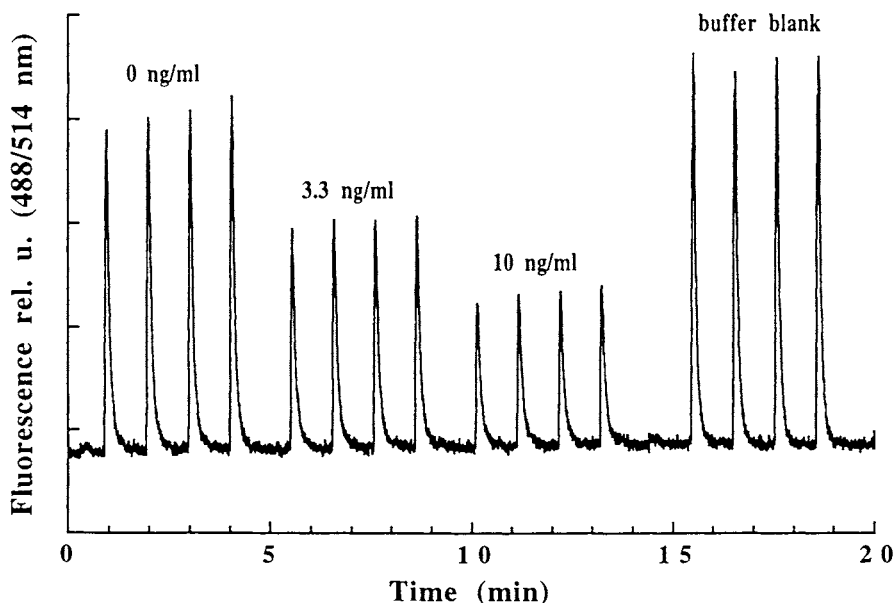
Fig. 9.15. Biological applications of the flow immunoassay without any clean-up step, before analysis of urine (U0, U3, U10) where 0, 3 and 10 correspond to the spiked concentration (ng/ml atrazine): 0 ppb, corresponds to an unspiked 0.01 M PBS sample.

atrazine in 0.01 M PBS buffer (see Fig. 9.16). To remove these matrix effects and achieve lower detection limits, a trace-enrichment step was applied, based on solid-phase extraction with the highly biocompatible RA phase, as in work outlined in a previous paper [90].
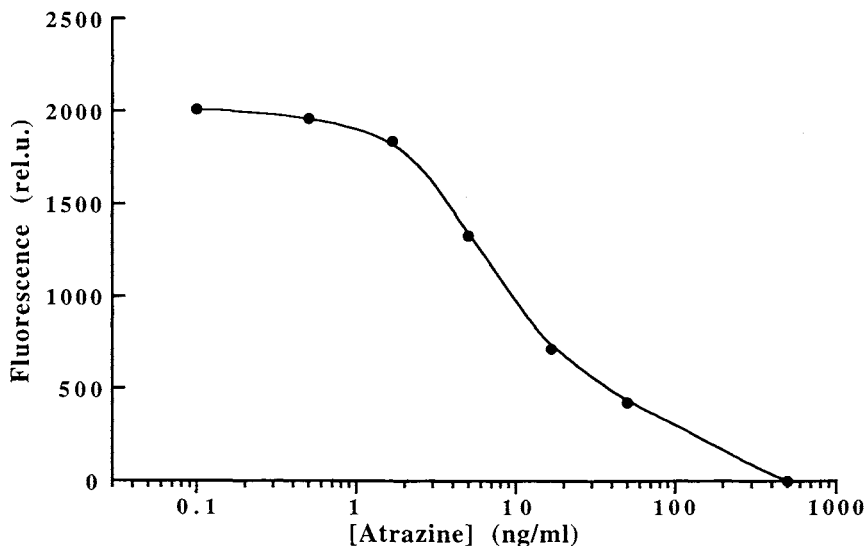
Fig. 9.16. Calibration plot of spiked blood samples diluted and then enriched using a solid-phase extraction (SPE) step before analysis with the flow immunoassay system.

The breakthrough volumes (defined at 5% breakthrough of total response) were found to be high for both simazine and atrazine, at 30 ml and 50 ml, respectively. Plasma-protein binding of both atrazine, simazine and the transformation products could be cleaved efficiently, and recovery data close to 100% were obtained. The resulting detection limits for atrazine in plasma, and also for water samples, using this clean-up and trace-enrichment procedure were found to be 2 ng/ml and 20 pg/ml, respectively. The analyses could be performed at a sample throughput rate of 400 per 6 h working shift, and have been performed during an investigation period of one month with the optimized system. General principles are outlined which should, because of the relatively low specificity of the RA-phase, be applicable to any type of antigen with a molecular weight ≤ 3000 Da, as long as the AbAg–AbAg* kinetic reaction conditions are favorable.

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Chapter 10

HPLC methods for the determination of mycotoxins and phycotoxins

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CONTENTS

10.1 INTRODUCTION

Mycotoxins are generally considered to be toxic substances produced by certain types of fungi which grow on plants of agricultural importance either before harvest or during storage. Some (such as aflatoxins) are of enough concern that they require regulatory

monitoring in plant material destined for human consumption or as animal feed. Phyco-toxins are potentially toxic substances which are produced by marine phytoplankton and which may accumulate in shellfish or finfish through the food chain. Certain phycotoxins such as saxitoxin are acutely toxic to humans and their presence in shellfish has been known for many years. In recent years, it has been recognized that phycotoxins now represent a worldwide concern. It has become extremely important for regulatory agencies throughout the world to ensure that seafood products destined for human consumption are safe. In order to ensure human safety, analytical methods are required that will enable the routine monitoring of food products for both mycotoxins and phycotoxins.

High-performance liquid chromatography (HPLC) has become an extremely important technique for the determination of mycotoxins and phycotoxins in biological samples. Indeed for mycotoxins, extensive research employing HPLC has been reported for many different classes of these substances. Phycotoxins, however, have received much less attention in this regard, mainly due to the difficulty in isolating and identifying the substances causing toxic responses and due to the general lack of standards for most classes of these compounds. However, the need for chemical methods for phycotoxins is becoming increasingly important due to the general trend away from using mammalian bioassays as well as the great increase in culturing fish and shellfish for human consump-tion thus requiring increased regulatory monitoring. The chemical structures of many mycotoxins and phycotoxins lend themselves to analysis by HPLC rather than gas chro-matography or other instrumental techniques. As a result, HPLC has become well exploited for determining these types of compounds.

Research on analytical methods for both mycotoxins and phycotoxins continues at a good pace. Methods for mycotoxins have been the subject of several reviews [1–11] all of which deal with HPLC completely or in part. There have been a number of recent reviews of analytical methods for phycotoxins [12–15] that have included HPLC. The aim of this chapter, which updates an earlier chapter published in 1993 [16], is to present the latest developments in the determination of mycotoxins and phycotoxins by HPLC. Included in this chapter are novel chromatographic systems, sample preparation techniques, the use of chemical derivatization and HPLC–mass spectrometry (MS). Emphasis is placed on the latest research that has been reported.

10.2 MYCOTOXINS

10.2.1 Aflatoxins B_1, B_2, G_1 and G_2

Aflatoxins (see Fig. 10.1 for structure) are secondary metabolites produced by *Asper-gillus flavus* and *A. parasiticus* types of molds which grow on many varieties of cereal grains and nuts. These substances have been shown to be both toxic and carcinogenic in many animal species including man [17,18]. Because of this, active research on sensitive, rapid and reliable methods for their analysis continues. HPLC with fluorescence detection has been used for many years for the determination of aflatoxins [19–21]. Recent research has focused primarily on improving sensitivity through derivatization reactions or selec-tive detection systems such as HPLC–MS. In addition, much work has been reported on improved sample clean-up enabling the detection of lower concentrations of these chemi-cals.
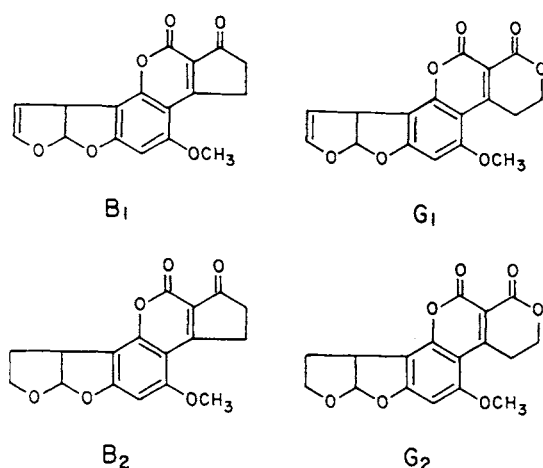
Fig. 10.1. Structures of aflatoxins B_1, B_2, G_1 and G_2.

10.2.1.1 Post-column reactions

In the HPLC determination of aflatoxins B_1, B_2, G_1 and G_2 using fluorescence detection, only B_2 and G_2 fluoresce with enough intensity in aqueous-based mobile phases to be analytically useful. However, it was observed that reaction of aflatoxins B_1 and G_1 with a variety of reagents such as strong acids [22] or the oxidants, chloramine T [23], bromine [24] or iodine [25–30] led to a significant increase in fluorescence intensity. The iodine reaction has been optimized for enhancement of aflatoxin B_1 [25] and applied to feedstuffs, corn and pet foods [26–29]. The product of the reaction appears to be an iodine-methoxy addition compound as shown in Fig. 10.2 [31]. The post-column reaction involves mixing the HPLC effluent with saturated aqueous iodine solution and heating at 70°C. The resulting derivatives could be detected in sub-nanogram quantities enabling the detection of ng/g levels in foods and feedstuffs. A modification of this procedure involved the use of a solid phase iodine reservoir [32].

Electrochemically generated bromine has been successfully used for aflatoxin analysis [33–35]. Bromine is generated from bromide present in the mobile phase by passage of the column effluent through an electrochemical cell. The amount of bromine produced can be controlled by the generating current. This approach has several advantages over the iodine post-column reaction and is applicable to routine analyses of samples for aflatoxins in the
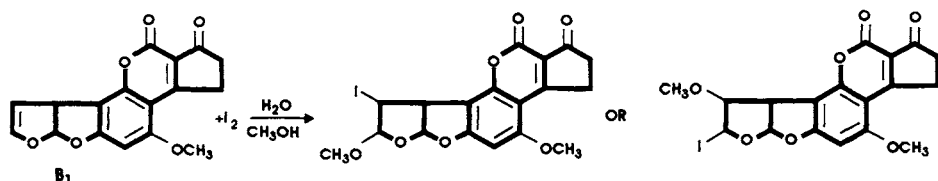
Fig. 10.2. Reaction of aflatoxin B_1 with iodine/methanol to yield iodomethoxy addition compounds.

low ng/g range. Post-column derivatization with pyridinium bromide perbromide has been used to determine aflatoxins in spices [36].

Automation of extraction and clean-up procedures as well as full method automation have been used for post-column derivatization methods [30,35]. Interlaboratory collaborative studies have also been carried out [37,38].

The post-column addition of β-cyclodextrin to enhance the fluorescence of B_1 and G_1 was evaluated for the determination of aflatoxins in corn [39]. The fluorescence intensities of aflatoxins B_1 and G_1 were greatly enhanced apparently due to the formation of inclusion complexes with β-cyclodextrin. Sub-nanogram quantities of the toxins could be detected. The interaction has been studied thermodynamically and 1:1 stoichiometry suggested [40]. When other cyclodextrins were used as post-column reagents, the increase in fluorescence was greatest for heptakis-2,6-β-O-dimethyl-cyclodextrin [41].

Photochemical derivatization also increased the fluorescence response of aflatoxins B_1 and G_1 with probable hydration of the furan double bond [42].

10.2.1.2 HPLC–MS

Thermo-spray (TSP) MS coupled to HPLC has been evaluated for the confirmation of aflatoxins and applied to a variety of products [43–45]. Hurst et al. [43] evaluated the technique for the direct confirmation of aflatoxins B_1, B_2, G_1 and G_2 without the need for derivatization. The optimum conditions employed TSP with 'filament on' or in the 'discharge' ionization mode. Picogram quantities of the aflatoxins could be detected with selected ion monitoring.

In a study of the bisulfite addition compounds of aflatoxins B_1 and G_1 [44], it was found that HPLC-TSP MS could be used to advantage to confirm these substances in a variety of plant extracts. Sodium bisulfite reacts with aflatoxins B_1 and G_1 to form bisulfite adducts across the double bonds of the furofuran ring. Aflatoxin M_1 and aflatoxicol also yield bisulfite adducts. Mass spectrometric responses obtained in the positive ion mode (either with ion-evaporation or 'filament on' chemical ionization) were 10–20-fold more intense than with negative ion conditions. Using ion-pair chromatography, the bisulfite adducts of aflatoxins B_1 and G_1 were separable from aflatoxins B_2 and G_2 which were unaffected by the bisulfite treatment. Applications to samples such as corn, milk and liver demonstrated that the method was useful, although for aflatoxin B_1 in corn the bisulfite derivatization method was about five times less sensitive than direct HPLC-TSP MS of the underivatized extracts, which could detect as low as 20 ng/g B_1.

A micro-flow particle beam interface was used to determine aflatoxins in peanut meal by HPLC–MS [46]. HPLC–MS/MS has also been explored [47].

10.2.1.3 Other chromatography and detection work

Tutelyan et al. [48] employed a normal-phase HPLC-fluorescence detection procedure with a mobile phase consisting of diethyl ether/methanol/water combinations and obtained good resolution of aflatoxins B_1, B_2, G_1, G_2 and M_1 without pre- or post-chromatographic derivatization. They employed a flow-cell packed with silica gel similar to that used elsewhere for normal-phase HPLC of these compounds [49]. This method was successfully used to analyze over 4500 plant foodstuffs for aflatoxins. Detection limits were

reported to be as low as 100 ng/kg. Normal-phase micro-HPLC, featuring axial illumination and elimination of the mobile phase by nebulization at the outlet orifice of the capillary, was applied to determination of aflatoxin standards using laser-induced fluorescence [50].

Differential-pulse amperometric detection using a dropping mercury electrode was evaluated for the detection of aflatoxins separated by HPLC [51]. It was found that although each of the four aflatoxins (B_1, B_2, G_1, G_2) yielded about the same sensitivity under optimum potential conditions for each individual compound, a compromise potential was required so that the four could be determined together. The detection limits for the individual aflatoxins were similar, being about 5 ng per injection. An advantage of this approach is that aflatoxins B_1 and G_1 do not require derivatization or other special treatment to improve their sensitivity as is required with the fluorometric detection methods.

Capillary micellar electrokinetic chromatography was compared to conventional normal and reverse-phase chromatography for the separation and detection of aflatoxins B_1, B_2, G_1 and G_2 [52]. The technique offers potential in terms of chromatographic efficiency and speed of analysis. However, it has not been applied to quantitative sample analysis on a routine basis and the limit of detection was only about 1 μg/g in corn meal.

10.2.1.4 Improved sample clean-up for HPLC

Recent advances in the sample clean-up of extracts for aflatoxin analysis have mainly focused on solid phase extraction (SPE) using small disposable cartridges filled with a variety of adsorbents which selectively retain the aflatoxins or sample co-extractives. Silica gel [53] has been used by several research groups for purification of plant extracts prior to HPLC determination. Hutchins et al. [53] thoroughly evaluated silica SPE cartridges for aflatoxin determination and found that under optimum conditions and using conversion of B_1 and G_1 to their respective hemiacetals, they could detect less than 1 ng/g aflatoxin B_1 in corn with very good repeatability over a wide range of concentrations. The method involved passing a chloroform/hexane extract of corn through a silica SPE cartridge which retained the aflatoxins. The cartridge was rinsed with hexane/chloroform (3:1) before the aflatoxins were eluted with hexane/acetone (1:1). This fraction was clean enough to derivatize and analyze by reverse-phase HPLC with fluorescence detection.

A phenyl-bonded SPE cartridge was successfully employed for the clean-up of groundnut meal extracts [54]. In this case, an aqueous sample extract was mixed with water, acetic acid and a lead acetate solution and then passed through a preconditioned phenyl bonded SPE. The aflatoxins were retained and the cartridge washed with water before the compounds of interest were eluted with a small volume of chloroform. The chloroform fraction was then evaporated to dryness for conversion of B_1 and G_1 to their hemiacetals before reverse-phase HPLC analysis with fluorescence detection. This method could detect low ng/g levels of aflatoxins B_1 and B_2 in groundnut meal.

A novel multifunctional type of SPE cartridge has been evaluated for a variety of plant extracts [55,56]. The packing material consists of both nonpolar and polar (charged) active sites. The nonpolar sites remove fats and other lipophilic materials while the charged sites (which consist of both dipolar and anion exchange sites) remove proteinaceous substances, carbohydrates and other polar co-extractives. These processes are carried out simulta-
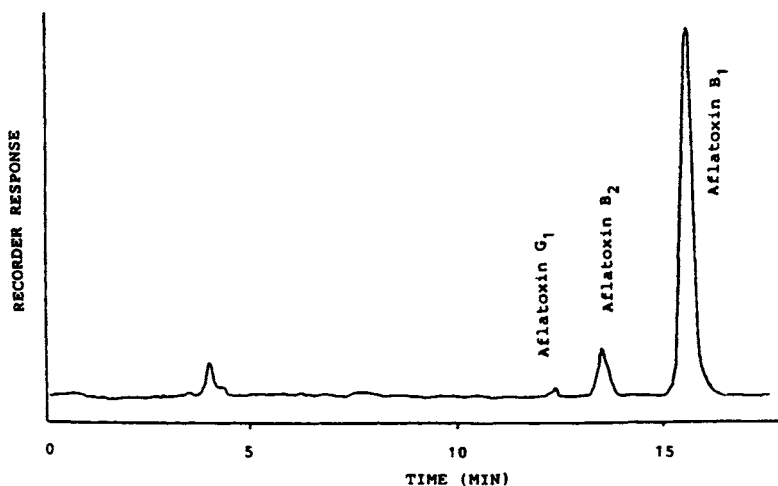
Fig. 10.3. HPLC chromatogram of aflatoxins B_1, B_2 and G_1 in dried figs. Concentrations: 11.5 ng/g B_1, 1.1 ng/g B_2 and 0.6 ng/g G_1. Reverse-phase chromotography with post-column iodination and fluorescence detection. (Reproduced from Ref. [60] with permission of Taylor & Francis, London, UK.)

neously in a single passage of the plant extract through the packing material. The detection limits were less than 0.5 ng/g for aflatoxins B_1, B_2, G_1 and G_2 by reverse-phase HPLC with fluorescence detection. The method has been studied collaboratively and has been adopted as an official method by AOAC International [57].

Immunoaffinity columns are becoming increasingly popular for cleaning up plant extracts for aflatoxin determination by HPLC [58]. There are now a variety of such columns commercially available. They consist of an anti-aflatoxin antibody bound to a gel material packed into a plastic cylinder or cartridge. When an extract is passed through the packing, the aflatoxins bind to the immobilized antibody while other sample co-extractives are washed through the column. The aflatoxins are then recovered by elution of the column with methanol or acetonitrile which liberates them from the antibodies. Because of the extremely selective nature of the clean-up, this type of column has found use for the analysis of aflatoxins by HPLC in a variety of plant products, including nut products and dried fruit [37,59–62], and other foods and animal feed [37,62–64]. Fig. 10.3 shows a chromatogram of an extract of dried figs containing low ng/g levels of aflatoxins B_1, B_2 and G_1 which had been cleaned up using immunoaffinity chromatography. As can be seen, the aflatoxins are easily detected at these levels.

A two-column SPE clean-up procedure for aflatoxin B_1 in cattle feed which has successfully passed an interlaboratory study [65] has been automated [66]. The method involves SPE clean-up on a Florisil cartridge followed by a reversed-phase C_{18} cartridge and injection of the cleaned up extract into an HPLC system with post-column bromination and fluorescence detection. The fully automated procedure compared well with the manual method in terms of repeatability, but the recovery of B_1 at low ng/g levels was only about 40%. The method does appear suitable for screening purposes.

Gel permeation chromatography (GPC) has been evaluated for the clean-up of extracts
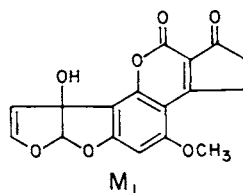
Fig. 10.4. Structure of aflatoxin M_1.

of cereals and animal feed [67]. The method involves extraction of the aflatoxins with dichloromethane/water (10:1) followed by GPC using dichloromethane/hexane (3:1) as eluent. The fraction containing the aflatoxins is concentrated and then analyzed by reversed-phase HPLC. Aflatoxin levels as low as 1 ng/g could be quantitated.

10.2.2 Aflatoxin M_1

Aflatoxin M_1 (Fig. 10.4) is a hydroxylated metabolite of aflatoxin B_1 and is of concern because significant levels of it can occur in milk of animals which have been fed aflatoxin B_1 contaminated feed. Much work on the determination of aflatoxin M_1 in dairy products by a variety of methods has appeared in the literature during the past decade and has been reviewed [68]. Since then new developments continue to be reported for aflatoxin M_1 determination in dairy products.

Aflatoxin M_1, like B_1 and G_1, has been found to fluoresce more intensely after derivatization. The reaction conditions for the conversion of M_1 to M_{2a} using trifluoroacetic acid (TFA) have been studied in detail [69]. However, aflatoxin M_1 does itself fluoresce enough to enable its direct determination in dairy products depending upon the effectiveness of the sample clean-up.

Immunoaffinity column clean-up of extracts of dairy products for HPLC analysis has been evaluated using both off-line [58,70,71] and on-line [58,72–74] procedures. The off-line procedure for milk involved only a centrifugation step to remove fat before the milk was passed through the immunoaffinity columns [70]. More than a liter of milk could be passed through a single column without difficulty. HPLC analysis involved reversed-phase chromatography with direct fluorescence detection. The affinity column clean-up was extremely effective, resulting in detection limits of about 50 pg/l. Application of the same type of affinity column to cheese samples required extraction and liquid–liquid partitioning before the sample extract could be loaded onto the column [71]. Although not as simple as the affinity column clean-up for liquid or powdered milks, the method was superior to other procedures for aflatoxin M_1 in cheese. The detection limits were about 5 ng/kg using the same HPLC-fluorescence detection system as for milk. An interlaboratory study on an immunoaffinity column method for milk powder with off-line HPLC showed reproducibility of 11–23% in the tested range of 80–600 ng aflatoxin M_1/g [76].

The on-line affinity column clean-up-HPLC analysis involved column switching first to load and wash the affinity column before elution and second, transferring the aflatoxin M_1 fraction to a reversed-phase pre-column for collection and preconcentration of the toxin before switching to the analytical column [72–74]. One of the main difficulties with this approach was that after a few sample analyses, the affinity column lost its effectiveness.

An on-line dialysis unit has been evaluated as an additional clean-up before the affinity column [74]. Although repeatability of the fully automated system was good, the overall recovery was extremely low (about 6%). Detection limits were reported to be about 10 ng/l.

Other automated systems incorporating column switching for on-line dialysis followed by reversed-phase enrichment of aflatoxin M_1 and HPLC with fluorescence detection have been evaluated for milk samples [75,77]. Recoveries with a dialysis procedure were about 50%. Detection limits were in the range of 20–50 ng/l.

Automated methods incorporating a laboratory robot have been developed for aflatoxin M_1 in milk [78,79]. The robot operated an extraction station which involved sample application to an SPE or immunoaffinity column, washing and elution. The fraction containing aflatoxin M_1 was transferred to an automated HPLC system, which included an on-line preconcentration unit if necessary, for determination using reversed-phase chromatography with fluorescence detection. The systems could handle 16–32 samples in an 8-h work period and the methods could measure 0.1 μg/l aflatoxin M_1 in milk.

In a comparison study between an enzyme-linked immunosorbent assay (ELISA) and an immunoaffinity column HPLC method [80], the former proved unreliable at levels close to 50 ng/l.

10.2.3 Ochratoxin A

Ochratoxin A (structure, Fig. 10.5) is a nephrotoxic and carcinogenic mycotoxin that is highly toxic to several animal species. It is produced by fungi of the *Aspergillus* and *Penicillium* genera which infect cereal grains and other plants used as human food or animal feed. Because of this, much research has been reported in the literature on method development, particularly by HPLC, so that surveillance data could be generated on the presence of ochratoxin A in food and animal feed. Also, since animals can retain detectable amounts in their blood and certain tissues, there is a need to have methods capable of monitoring animal products as well.

Analytical methods (including TLC, HPLC, spectrophotometry and immunoassay) for ochratoxin A have been reviewed [81]. Reversed-phase HPLC with an acidified mobile phase and fluorescence detection has been employed for the determination of ochratoxin A in cereal grains [82–85], animal feed [83,86], pet foods [28], kidney [82] and human urine [87]. These methods employed an initial organic solvent extraction followed by SPE or open column chromatography clean-up before analysis by HPLC. This approach successfully passed interlaboratory collaborative study [82,84]. Detection limits were in the low ng/g range for the cereals and kidney and in the ng/l range for human urine.

Ion-pair HPLC has been successfully used for determination of ochratoxin A in coffee products [88], human plasma [89] and cheese [90]. In this method, the mobile phase was
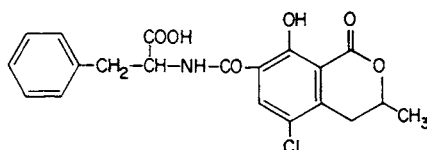
Fig. 10.5. Structure of ochratoxin A.

made basic (pH 7.5–9) or acidified (pH 5.5) and low millimolar concentrations of ion pairing agents such as cetyltrimethylammonium bromide, tetrabutylammonium bromide or tetrabutylammonium hydroxide were added. Detection limits for coffee products were in the sub-ng/g range when affinity chromatography clean-up was employed [88]. The detection limits for the ion-pair HPLC plasma method were 0.02 ng/ml [89]. In the method for cheese, terbium chloride was included in the post-column reagent for time-resolved luminescence, which gave greater sensitivity than direct fluorescence and detected citrinin also [90].

β-Cyclodextrin as a mobile phase additive resulted in a slight increase (15%) in fluorescence intensity of ochratoxin A [85].

Immunoaffinity column clean-up has been shown to provide very clean extracts for determination of ochratoxin A in coffee [88,91–95], cereals [58,96], beer [58], wine [97], grape juice [97], kidney [96] and blood plasma [98,99] by HPLC [58]. In a comparison of affinity chromatography and reversed-phase SPE chromatography for clean-up of extracts of the coffee products mentioned above, the affinity chromatography clean-up yielded about a fourfold improvement in detection limits. Affinity chromatography has been employed in an automated HPLC technique for ochratoxin A in cereal and animal products [100]. The affinity column clean-up was carried out either manually or with a commercial automated sample preparation system similar to that used for aflatoxins [61]. The authors found that the immunoaffinity approach was significantly faster than methods employing adsorption type chromatographic clean-up. The method was particularly attractive since the selectivity of immunoaffinity clean-up led to very clean chromatograms and unambiguous identification and quantitation of ochratoxin A at sub-ng/g levels in cereals and pig kidney. Fig. 10.6 shows chromatograms of extracts of wheat and pig kidney containing low ng/g levels of the toxin. The HPLC separation involved reversed-phase chromatography with an acidified mobile phase and fluorescence detection (333 nm excitation, 477 nm emission). Linkage of aflatoxin and ochratoxin A immunoaffinity columns for determination of these mycotoxins in pet foods required a compromise in HPLC conditions [28].

To confirm positive findings of ochratoxin A, several researchers have used chemical derivatization [87–89,98]. The reactions involved esterification of the carboxylic acid moiety of the toxin using either H_2SO_4, BF_3 or, more conveniently, HCl catalyzed reaction with methanol, ethanol or propanol. The resulting esters were determined using the same
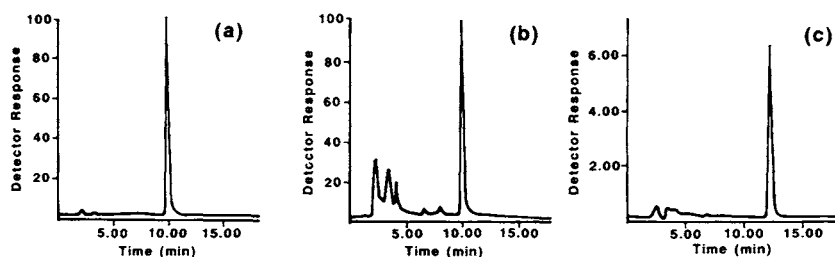
Fig. 10.6. HPLC chromatograms of ochratoxin A. (a) Standard equivalent to 20 ng/g. (b) Naturally contaminated wheat containing 13.7 ng/g. (c) Pig kidney spiked with 10 ng/g. (Reproduced from Ref. [100] with permission of Elsevier, Amsterdam.)

reversed-phase HPLC system as for unesterified toxin and were more strongly retained on reversed-phase columns. Another means of confirmation is the addition of carboxypepti-dase A to ochratoxin A in pig kidney extracts [101].The hydrolysis product ochratoxin α is formed, which eluted before ochratoxin A, although amounts produced were higher than expected from the ochratoxin A detected, suggesting the presence of other forms of ochratoxin A in the kidney [101]. HPLC–MS and HPLC–MS/MS have been evaluated for confirmation of ochratoxin A in barley and plasma extracts, respectively [102,103]. For the barley extracts, a direct liquid introduction (DLI) interface was employed with a quadrupole mass spectrometer [102]. The HPLC effluent, consisting of aqueous acetoni-trile acidified with formic acid, was split so that only 2.5% of the mobile phase entered the DLI interface. Using negative ion chemical ionization and monitoring at m/z 403 (M^-), sub-ng/g levels of ochratoxin A were detectable. HPLC-electrospray MS/MS could detect low pg quantities of ochratoxin A and sub-ng/ml in plasma using multiple reaction moni-toring and ochratoxin B as internal standard [103].

10.2.4 Fumonisins

The fumonisins (structures, Fig. 10.7) are a group of mycotoxins produced by *Fusarium moniliforme* and *F. proliferatum*, which are common fungal contaminants of corn. Because of their toxicity and carcinogenicity, there is a need for sensitive, accurate methods for their analysis in corn products destined for human consumption. A large number of HPLC methods for fumonisins have been reported in the literature. Since fumonisins have no analytically useful UV absorption or fluorescence, they must be derivatized to products which are detectable using standard HPLC detectors. Thus the methods reported to date involve UV [104,105] or fluorescence [104–124] derivatization with reagents such as maleyl anhydride [104,105], *ortho*-phthaldialdehyde (OPA) and 2-mercaptoethanol [106,109,120–122], OPA and 2-methyl-2-propanethiol [110], OPA and *N*-acetyl-L-cysteine [123], fluorescamine [104,111,113,124], 4-fluoro-7-nitrobenzofura-zan (NBD-F) [107,108,113], 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadia-zole (DBD-F) [112], naphthalene-2,3-dicarboxaldehyde (NDA) [115,116], 9-fluorenylmethyl chloroformate (FMOC) [114], 6-aminoquinolyl *N*-hydroxysuccinimidyl-carbamate [118,119] and fluorescein 5-isothiocyanate (FITC) [117]. The methods employ-ing OPA, NBDF and NDA appear to offer the most in terms of method simplicity and
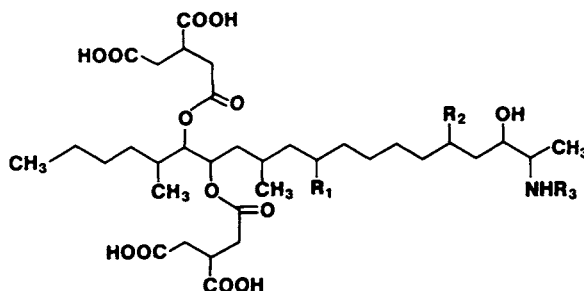
Fig. 10.7. Structures of fumonisin B_1 ($R_1 = R_2 = OH$, $R_3 = H$), fumonisin B_2 ($R_1 = H$, $R_2 = OH$, $R_3 = H$), fumonisin B_3 ($R_1 = OH$, $R_2 = R_3 = H$), fumonisin B_4 ($R_1 = R_2 = R_3 = H$), fumonisin A_1 ($R_1 = R_2 = OH$, $R_3 = CH_3CO$) and fumonisin A_2 ($R_1 = H$, $R_2 = OH$, $R_3 = CH_3CO$).

detection limits. Since the OPA/2-mercaptoethanol derivative gives the least stable derivative yet is the most commonly used reagent, work has been done to optimize the reaction conditions [109,120–122]: a reaction time of 1 min is generally used. In most cases derivatization is pre-column; however, OPA/*N*-acetyl-L-cysteine was used post-column [123].

Fumonisins have also been determined by HPLC with techniques that do not involve derivatization. An evaporative light-scattering detector could detect about 60 ng fumonisin B_1 per injection after separation by HPLC on a base-deactivated C_8 column and a mobile phase consisting of a trifluoroacetic acid (pH 2.7)/ acetonitrile gradient [125,126]. This was used for analysis of purity of the fumonisin B_1 and, with a semi-preparative column, for purification of analytical standards.

HPLC–MS procedures have received considerable attention recently. HPLC-fast-atom bombardment (FAB) MS [124,127,128] and HPLC-electrospray MS [129–133] have been used for quantitation, confirmation, purity determination, discovery of new fumonisins and detection of structural isomers of partially hydrolyzed fumonisin B_1. HPLC–MS has been used for the confirmation of fumonisin B_1 in cultures of *Alternaria alternata* f. sp. *lycopersici* [134]. Tetramethyl esters of fumonisins prepared with diazomethane were characterized by particle-beam HPLC–MS [135]. Recently, HPLC–MS/MS has provided highly structure-specific qualitative and quantitative determination of fumonisins [136–139]. It has been applied to determination of fumonisins B_1 and B_2 in corn and corn foods [136,137], characterization of impurities in fumonisin B_1 [138], identification of new
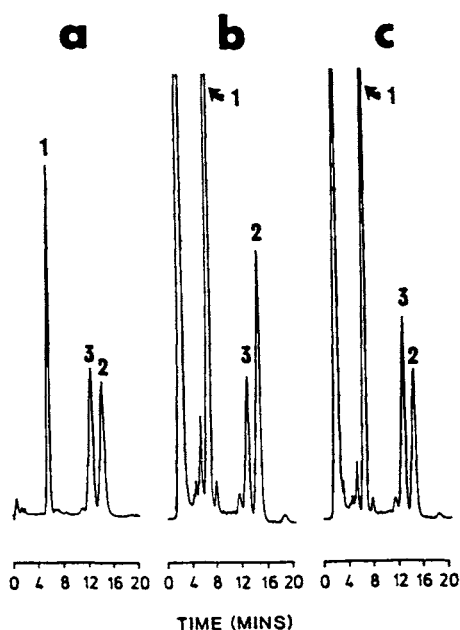
Fig. 10.8. Chromatograms of (a) OPA-mercaptoethanol derivatives of 50 ng of fumonisin B_1 (1), B_2 (2) and B_3 (3); (b) derivatized extract of corn-based feed sample (8 μg/g B_1, 4.1 μg/g B_2 and 2.7 μg/g B_3); (c) as in (b) but spiked with standard B_3. (Reproduced from Ref. [109] with permission of AOAC International, Arlington, VA).

fumonisins in *Fusarium* cultures [139], and to obtain MS/MS spectra from a single chromatographic run of the four monoesters, six diesters, four triesters and one tetraester formed by incomplete acid catalyzed methylation of fumonisin B_1 [138].

Methods mostly use methanol/water or acetonitrile-water for extraction of corn samples followed by strong anion exchange (SAX) or C_{18} SPE or immunoaffinity column clean-up of the extracts. The methods have been applied to analysis of corn and corn foods, milk, beer and biological fluids [140–147]. In the case of the SAX column, fumonisins were eluted with methanol/acetic acid and the fraction evaporated to dryness before derivatization. LC separations were carried out by reversed-phase chromatography using slightly acidified mobile phases (pH 3.5–5). Detection limits using OPA as the fluorescent derivatization reagent were about 50 ng/g for fumonisin B_1, and about 100 ng/g for fumonisin B_2; lower detection limits could be achieved with immunoaffinity column clean-up [58]. The method for corn employing SAX clean-up and OPA derivatization has been collaboratively studied and adopted as an official method of AOAC International [148–150]. The NBD-F and NDA methods could detect fumonisins B_1 and B_2 in the 100 ng/g range [107]. Fig. 10.8 shows chromatograms of an extract of a corn-based feed sample containing low μg/g levels of fumonisins B_1, B_2 and B_3 after conversion to the OPA derivatives. Fig. 10.9 shows examples using NBD-F as derivatizing reagent. It is also possible to determine the hydrolysis product of fumonisin B_1 (HFB$_1$ or AP$_1$) in alkali processed corn foods [151,152]. Comparative study of HPLC and ELISA for determination of fumonisins in corn foods indicates good correlation but generally higher estimates by
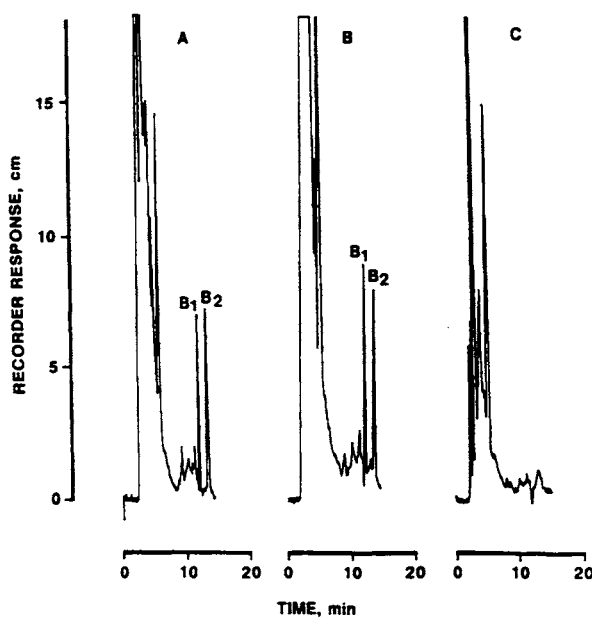
Fig. 10.9. Chromatograms of NBD-F derivatized extracts of (A) corn meal spiked with 2.5 μg/g each of fumonisins B_1 and B_2. (B) Spiked corn meal kept at 22°C for 1 h, and (C) spiked corn meal after heating at 220°C for 25 min.

ELISA [153,154]; with beer, however, ELISA appeared to underestimate compared to HPLC [145].

10.2.5 Trichothecenes

Trichothecenes (structures, Fig. 10.10) are mycotoxins produced by *Fusarium* spp. and other types of fungi which can contaminate grains and other plants. There has been a large amount of work reported in the literature on the analysis of these substance by various chromatographic means, including HPLC. This area has been reviewed [155]. For deoxynivalenol (DON), UV detection at 220 nm is fairly sensitive using charcoal containing clean-up columns; the detection limit for white flour was 20 ng/g [156] and for milk was 5 ng/ml [157]. A multifunctional clean-up column (MycoSep^{TM}) is also proving of value in the determination of DON in grains [158].

Recent HPLC research on detection of trichothecenes has involved evaluation of a variety of derivatization reagents to make the mycotoxins more detectable by fluorescence or UV absorption. Reagents such as coumarin-3-carbonyl chloride [159] and anthracene-9-carbonyl chloride [160] have been successfully studied for several trichothecenes. These reagents react with the hydroxyl substituents to form fluorescent products which are separable by reversed-phase HPLC. Low nanogram quantities of T-2 toxin, for example could be detected. These methods have not, however, been evaluated for routine detection in plant materials.

Diphenylindenone sulfonyl (Dis) esters of several trichothecenes that do not have a UV chromophore, including T-2 toxin, HT-2 toxin, diacetoxyscirpenol, T-2 triol, T-2 tetraol and iso-HT-2 toxin, were prepared for both TLC and HPLC analysis [161]. For HPLC, UV detection at 278 nm was employed. However, the minimum detectable quantities per injection were 30–50 ng which is not sensitive enough for residue determinations in contaminated grains or other plants. The authors found the method particularly suitable for in vivo pharmacokinetic studies involving T-2 triol and T-2 tetraol.
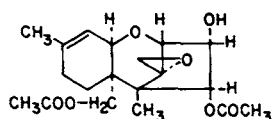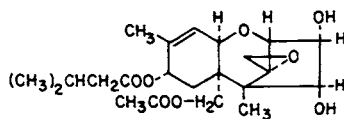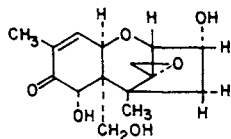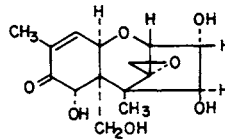
Fig. 10.10. Structures of four trichothecene mycotoxins.

Post-column treatments of the effluent by means of photolysis [162] or with alkali followed by reaction with methyl acetoacetate/ammonium acetate [163] have been evaluated for DON, nivalenol and fusarenon-X. The photolysis method involves passing the HPLC effluent through a post-column UV irradiation unit which converts the trichothecenes to oxidizable products that can be detected by oxidative amperometric detection. As low as 1–2 ng of DON could be detected. The advantage of this type of derivatization is that no post-column reagent addition is required. The alkali–methyl acetoacetate reaction appears to be somewhat less sensitive and considerably more complex requiring two reagent pumps and reaction coils. However, with suitable clean-up, DON and nivalenol could be detected in corn, wheat and barley at 0.05–1 μg/g concentration levels.

Direct reductive electrochemical detection (− 1.4 V) has been studied for application to the HPLC analysis of DON in corn, rice and wheat products [164]. Sub-nanogram quantities could be detected and, with the extraction and clean-up procedure employed, sub-μg/g levels of DON could be quantitated in grain samples. The method, however, was not applicable to the type A toxins (see Fig. 10.10) since they were electrochemically inactive under the conditions used.

An HPLC column-switching technique has been developed for the determination of DON in maize and corn silage [165]. It involved heart-cutting from one reversed-phase C-18 column to a second. The method could detect 0.2 μg/g of DON using UV detection at 215 nm.

HPLC–MS has been shown to offer potential for trichothecene determination: TSP-MS, dynamic FAB-MS and plasma spray-MS have all been evaluated for a variety of trichothecene analogues [166–170]. The TSP mass spectra exhibit practically no fragmentation showing only an abundant ammonium adduct ion. Plasma spray, involving a somewhat more energetic ionization process than TSP, produces some fragment ions in addition to the same ammonium adduct ion as observed with TSP. Dynamic FAB yielded numerous fragment ions [170]. All three techniques appear suitable for monitoring trichothecenes in plant or animal tissues without need for derivatization. However, for structural identification, the dynamic FAB technique would be preferred because of the additional fragmentation obtained. HPLC–MS has not been studied extensively for residue analysis although the application of TSP-MS to the detection of several toxins (DON, T-2 toxin and diacetoxyscirpenol) in porcine plasma and urine has been reported [170]. Detection limits were about 1 ng per injection for the trichothecenes examined.

Supercritical fluid chromatography on HPLC columns with UV and MS detection was applied to the separation and identification of several trichothecenes in *Fusarium* culture extracts [171]. Supercritical fluid extraction has also now been investigated in a method to determine DON [172] and other trichothecenes [173] in grains by HPLC. Sensitivity was poor with UV detection if no clean-up column was used but low ng/g levels of DON, T-2 toxin and diacetoxyscirpenol were detectable by HPLC–MS/MS [173]. Detectability of DON was considerably improved if an immunoaffinity column was used for clean-up of the extracts [172].

10.2.6 Zearalenone

Zearalenone (structure, Fig. 10.11) and its metabolites, α- and β-zearalenol, are biologically active mycotoxins possessing estrogenic activity. There is also some evidence for
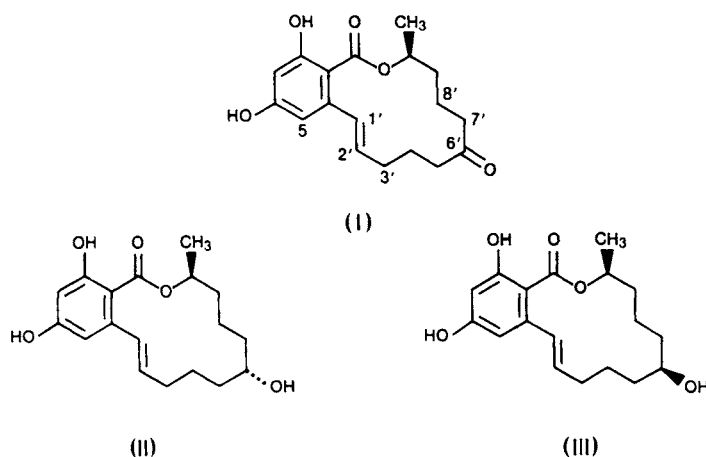
Fig. 10.11. Chemical structures of zearalenone (I), α-zearalenol (II) and β-zearalenol (III).

the carcinogenicity of zearalenone. This mycotoxin is produced by *Fusarium grami-nearum* and several other species of *Fusarium* fungi which can infect a variety of agricultural crops, particularly corn and other grains.

Recent HPLC methods for zearalenone determination have employed reversed-phase chromatography with direct fluorescence detection [174–179]. These methods used several different sample clean-up techniques. Liquid–liquid partitioning was used to purify aqueous extracts of biological fluids (milk, blood, urine, bile) from cows or swine [174]. Detection limits for zearalenone and the two zearalenols were in the low ng/ml range. Solid phase extraction using an aminopropyl column was found to be useful for clean-up of milk after an initial extraction with basic acetonitrile and partitioning into dichloromethane [175]. The partitioning was speeded up considerably using a hydrophilic matrix to absorb the aqueous phase after removal of acetonitrile by rotary vacuum evaporation. The clean-up enabled the detection of zearalenone and α-zearalenol in milk at levels as low as 0.2 ng/ml and for β-zearalenol, down to 2 ng/ml. GPC on SX-3 Biobeads was used to purify chloroform extracts of a variety of cereal and mixed animal feed samples for the determination of zearalenone [176]. Although useful for certain grains, the extracts of mixed feeds required additional clean-up using SPE silica columns. The detection limit using HPLC with an amino-bonded phase and fluorescence detection at 280 nm (excitation) and 470 nm (emission) was about 2 ng/g. In a method applied to laboratory cultures of *Fusarium graminearum* [177] and a method capable of determining both zearalenone and ochratoxin A in cereals and feeds [86], a silica cartridge was also used for clean-up. Other clean-up procedures used in methods for zearalenone are immunoaffinity column chromatography [178] and chromatography on piperidinohydroxypropyl Sephadex LH-20 gel [179].

The HPLC fluorescence detection of zearalenone in cereal extracts was modified by adding aluminum chloride solution to the column effluent in a heated post-column reactor [180]. The reaction with zearalenone resulted in a five-fold increase in fluorescence response. The nature of the reaction involved is still unknown. The authors found that this was particularly useful for determining low levels of zearalenone in cereals. However,
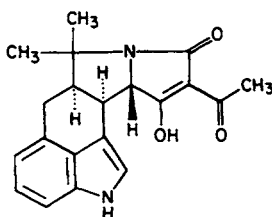
Fig. 10.12. Structure of α-cyclopiazonic acid.

in some cases, the matrix background in the chromatograms also increased substantially with the addition of aluminum chloride. Inclusion of β-cyclodextrin in methanol-water (pH 2–3) mobile phase allowed separation of zearalenone from ochratoxin A [85].

Electrochemical detection has been evaluated for the detection of zearalenone and zearalenol in corn [181]. The method involved chloroform extraction, liquid–liquid partition and reverse-phase HPLC with detection at +0.95 V using a glassy carbon working electrode. This system was capable of detecting as low as 20 pg per injection of the mycotoxins. Detection limits in corn were in the low ng/g range.

10.2.7 Cyclopiazonic acid

α-Cyclopiazonic acid (structure, Fig. 10.12) is a toxic mycotoxin produced by certain species of *Aspergillus* and *Penicillium*. It has been found in several plant products, such as corn and peanuts, as well as in *Penicillium* processed cheese. It is also known to accumulate in certain animal tissues as a result of the consumption of contaminated feed. Thus there is a need to monitor for the compound in plant and animal products which may be consumed by humans.

Several different HPLC methods have been evaluated for the determination of cyclopiazonic acid in corn peanuts, poultry tissue and culture media. These include ligand-exchange chromatography [182–185], normal-phase chromatography [186], normal-phase ion-pair partition chromatography [187] and ion exchange chromatography using an amino-bonded phase [188]. All methods employed UV absorbance detection at a wavelength between 279 and 284 nm or 225 nm [188]. The ligand-exchange methods made use of zinc acetate or zinc sulfate (as a chelating agent) to improve the peak shape when employing reversed-phase columns and aqueous mobile phases. The extraction and clean-up procedures mostly involved solvent extraction and liquid–liquid partition followed by SPE clean-up on silica. Quantitation limits were in the 50–100 ng/g range for corn, peanuts and poultry tissue using the ligand exchange systems [183,184].

10.2.8 *Alternaria* toxins

Alternaria mycotoxins, including alternariol, alternariol monomethyl ether, altenuene and tenuazonic acid have been determined by HPLC using several different extraction procedures and chromatographic systems [189]. The separations involved normal-phase [189,190] and reversed-phase [191–200] chromatography and where tenuazonic acid was included, ligand exchange, anion exchange and ion pair chromatography [201–205]. In most cases, UV detection around 256 nm, 280 nm or 340 nm was employed, although

fluorescence [189,197,203,204] and mass spectrometry [198] have also been used for detection. A detection limit of 0.05 ng for AOH and AME by fluorescence has been reported [204]. Another very sensitive LC determination procedure for AOH and AME (as well as altertoxins I and II) is electrochemical; 0.05 ng AOH was the detection limit [199]. This procedure involved dual in-series electrodes operating in the redox method where the toxins were oxidized (+1.0 V) by the first electrode, then the products detected at the second electrode at a negative potential (−0.1 V). The approach could detect several of the toxins at sub-μg/g levels in maize, rice and tomatoes infected by *Alternaria alternata* [192].

Methods were applied to laboratory cultures [192,194,201,202], rice leaves [201], tomatoes [196,204], grains [203], fruits [195,206] and other foodstuffs [206]. A sensitive LC method for AOH and AME in apple juice was recently developed by Delgado et al. [200] who used two solid phase extraction columns in series for clean-up followed by LC with UV detection at 256 nm. Detection limits were reported to be, respectively, 0.7 and 0.5 ng of standard and 1.6 and 0.7 μg/l apple juice. These are an improvement over a previous LC method for AOH and AME in apple juice, which had detection limits of 10 and 25 μg/l, respectively, by UV detection at 340 nm; extraction was with dichloromethane followed by silica gel column clean-up [195].

The structural isomers of the AAL toxins TA and TB – TA_1, TA_2, TB_1 and TB_2 – were separated as their OPA derivatives in a reversed-phase isocratic system [207].

10.2.9 Miscellaneous mycotoxins

HPLC has been used for the determination of a variety of mycotoxins as demonstrated above. The technique has also been applied to other types of mycotoxins although on a more limited scale due to the availability of better methods or to the fact that many of these compounds are not of much interest because of their limited presence in plant or animal products destined for consumption as food or animal feed.

Citreoviridin is a neurotoxic mycotoxin which may be present in corn or rice infected with certain *Penicillium* fungi such as *P. citreo-viride*. The toxin has been determined in corn and rice using organic solvent extraction, SPE clean-up on silica and amino columns and normal-phase HPLC with fluorescence detection (388 nm excitation and 480 nm emission) [208]. Minimum detectable concentrations were about 2 ng/g for corn.

Citrinin, a mycotoxin produced by several *Aspergillus* and *Penicillium* species, has been determined in cereals using normal-phase [209] or reversed-phase ion pair [210] HPLC with fluorescence detection. Ion-pair chromatography was required for the reversed-phase system to improve peak shape and column efficiency. The normal-phase system employed a novel acid-buffered silica column where the column was conditioned with aqueous citrate, then it was completely dried before passing the hexane–chloroform mobile phase through. Good peak shape and sensitivity were obtained using both methods either for fungal fermentations [210] or for cereals [209]. Low to sub ng/g levels of citrinin could be detected [209]. Post-column detection with 1 M hydrochloric acid greatly increased the fluorescence of citrinin eluted from a C_{18} column by an aqueous methanol mobile phase (pH 5.5) containing an ion-pairing reagent [211]. When citrinin is heated at 100–140°C in the presence of water a new toxin, citrinin H1, is formed [212]. The reaction was monitored by reversed-phase HPLC with UV detection at 254 nm.

Normal-phase HPLC with UV absorption has been used for the determination of the *Fusarium moniliforme* produced mycotoxin, fusarin C, in submerged cultures [213]. The method employed semi-preparative normal-phase HPLC to purify the culture extract followed by analytical HPLC with UV detection for quantitation. Three isomers were observed in the extracts due in part to decomposition under UV light. Interestingly, use of a C_{18} column with a chloroform–methanol mobile phase to separate fusarin C from its 8Z isomer has been reported [214,215].

HPLC has been used in combination with an ELISA immunoassay technique for the determination of another *Fusarium* mycotoxin, fusarochromanone (TDP-1), in barley, wheat and in cultures of rice and corn [216]. After initial extraction with methanol, the mycotoxin was isolated by reverse-phase HPLC and then acetylated and determined by ELISA. The detection limit was about 5 ng/g in barley and wheat compared to a detection limit of about 20 ng/g when ELISA was used without the HPLC purification. In other methods, applied to corn and wheat, clean-up on a silica cartridge or Florisil column coupled with HPLC (fluorescence detection) allowed detection of as little as 1–5 ng/g [217,218].

Moniliformin is a mycotoxin produced by a variety of *Fusarium* species. Several HPLC methods have been developed for its determination in cereals and fungal cultures [219–221]. These involved extraction with acetonitrile/water or water followed by clean-up on a combination of reverse-phase and SAX materials, SAX alone or DEAE Sephadex. The extracts could be analyzed by ion-pair or SAX HPLC with UV detection at 229 nm [219,220]. By reacting the moniliformin post-column with 1,2-diamino-4,5-dichloroben- zene at pH 11 to form a fluorescent derivative, a determination limit of 20 ng/g in cereals was achieved [221].

Several other *Fusarium* mycotoxins have also been determined by reversed-phase HPLC. Fusaproliferin was detected by UV absorption at 261 nm [222]. A sensitive method for determination of beauvericin in corn (detection limit 50 ng/g) was achieved when absorption at 192 nm in acetonitrile/water (90:10) was used [223]. Ammonia was added to the mobile phase to resolve the problem of tailing of the peaks of visoltricin (detected at 301 nm) and chlamydosporol isomers (detected at 210 nm) from *Fusarium* cultures [224].

Gliotoxin, a fungal toxin produced by *Aspergillus fumigatus* Fresenius and other *Aspergillus* species, has been determined in rice cultures and turkey lung tissues by reversed- phase HPLC with UV detection at 254 or 280 nm [225,226]. The chloroform extracts were cleaned up using GPC or silica SPE columns before HPLC determination. As low as 0.67 μg/g and 0.4 μg/g of gliotoxin could be determined in rice cultures and tissue, respec- tively.

Phomopsin A is a toxin produced by *Phomopsis leptostromiformis*, a fungus which can infect sweet narrow-leafed lupins often used as summer feed for sheep in Australia. A method employing HPLC has been evaluated for the detection of phomopsin A in lupin stubble [227]. The toxin is extracted with methanol-water, then subjected to liquid–liquid partition, followed by column clean-up on Amberlite XAD-2 and cation exchange chro- matography. The final purified extract is analyzed by reverse-phase HPLC with UV detection at 280 nm. As low as 0.5 μg/g could be detected.

Tremorgenic mycotoxins such as the paspalitrem and lolitrem groups are produced by several types of fungi including *Aspergillus, Claviceps* and *Penicillium*. Several paspali- trems have been determined (in cultures) by normal-phase HPLC with diode array UV

detection [228]. Lolitrem B (the major lolitrem toxin) has been determined in perennial rye grass by HPLC with fluorescence detection [229]. Fluorescence was found to be much more sensitive and selective than UV absorption for detection of lolitrem B in the rye grass samples. Fumitremorgin B was determined in corn at levels as low as 0.5 ng/g by reversed-phase HPLC with UV detection at 225 nm [230].

Ergot alkaloids are produced by the fungus *Claviceps purpurea* which can infect a variety of grains, particularly rye and the animal feed grass, tall fescue. Recent HPLC methods for ergot alkaloids include minor modifications [231,232] of the reversed-phase method first reported by Scott and Lawrence [233]. These methods were applied to the analysis of endophyte-infected tall fescue [232]. They made use of reversed-phase gradient HPLC with a slightly alkaline mobile phase and fluorescence detection. Detection limits for the various alkaloids were in the 50–200 ng/g range. A gradient ion-pair HPLC system using fluorescence detection was employed in a survey of cereals for a number of ergot alkaloids [234]. The ion pairing mobile phase improved peak resolution and led to a much longer column life compared to the alkaline mobile phase used earlier [233]. Detection limits were in the low ng/g range. Recently, HPLC-electrospray MS was used to identify ergovaline, ergosine, ergonine and other ergot alkaloids isolated from endophyte-infected tall fescue seeds [235].

Reversed-phase HPLC with diode array detection was used to identify the *Penicillium* mycotoxins isofumigaclavines A and B, roquefortine C and PR toxin in inoculated must and wine [236].

New methods for determination of sterigmatocystin in corn, bread and cheese have been developed that make use of HPLC–MS [237]; detection limits were about 2 ng/g for grains and grain products and 4 ng/g for cheese.

Patulin is a mycotoxin that is regulated at 50 μg/l in apple juice in several European countries. It can be determined by reversed-phase HPLC with UV detection at 254 nm or 272–276 nm [238–242]. Diode array UV detection has also been used [243]. Extraction is generally with ethyl acetate. Clean-up is by further extraction into sodium carbonate [238,239], by a diphasic dialysis membrane procedure and a silica cartridge [240], by a silica cartridge alone [241] or by a Florisil cartridge following extraction of patulin from the apple juice with toluene-ethyl acetate (75:25) on a hydrophilic matrix [242]. Detection limits of below 10 μg/l have been achieved, the lowest being 0.5 μg/l [240]. One of the methods has been studied collaboratively and adopted as an Official Method by AOAC International [238].

10.2.10 Multimycotoxin methods

A number of methods have been developed for the determination of a variety of mycotoxins in a single analytical scheme. Such methods are particularly useful because of the possibility of having several different mycotoxins present at the same time in certain crops. For example, a method for the extraction and detection of aflatoxins, ochratoxin A and zearalenone in grains, oilseeds and animal feeds using on-line sample clean-up and HPLC with post-column iodine treatment has been reported [244]. A method for detecting these same mycotoxins plus vomitoxin and secalonic acid D in grain dust was developed that used TLC for the detection of the aflatoxins and ochratoxin A and reversed-phase HPLC with UV detection for the remainder [245]. A multimycotoxin method for patulin,

penicillic acid, zearalenone and sterigmatocystin was developed for analysis of cocoa beans [246]. This method also used HPLC as the determinative step with simultaneous dual channel UV detection at 245 nm and 280 nm. GPC was used for clean-up of animal feed extracts for determination of aflatoxins, ochratoxin A and zearalenone by reversed-phase HPLC with fluorescence detection [247]. All of these methods could detect the mycotoxins at levels of concern.

Diode array UV absorbance detection has proven to be very useful for multimycotoxin screening by HPLC. Several studies have shown the potential of the technique for monitoring a wide variety of toxins. Isohata and Hayakawa [248] determined aflatoxins, ochratoxin A, citrinin and patulin by reversed-phase HPLC with diode array detection, and sterigmatocystin, zearalenone, deoxynivalenol and fusarenon-X by UV detection, with application to analysis of rice and corn. Frisvad and Thrane [249] reported on the HPLC-diode array UV characteristics of 182 mycotoxins and other fungal metabolites and the application of this technique to fungal chemotaxonomy. They used an alkylphenone retention index system for normalizing retention data. Similarly Paterson et al. [250] and Kuronen [251] evaluated HPLC with diode array detection for an extensive number of mycotoxins and fungal metabolites. This work has provided valuable information on the chromatography and absorption characteristics of many mycotoxins. Although it is unlikely that any one multimycotoxin procedure will be practical for the routine determination of such a wide variety of mycotoxins at levels of concern, these data indicate that in many cases multimycotoxin determination of selected toxins is very feasible.

HPLC–TSP MS has been used for the determination of a number of *Fusarium* mycotoxins [170] as well as a combination of toxins including several trichothecenes, patulin, zearalenone and ochratoxin A in a single chromatographic run [252]. In the latter case detection limits for the mycotoxins were in the 1–40 ng/g range in grain samples. HPLC–MS continues to demonstrate itself as a technique with much potential for routine determination and confirmation of mycotoxins in biological samples.

Micellar electrokinetic capillary chromatography was shown to resolve 10 mycotoxins – aflatoxins B_1, B_2, G_1 and G_2, sterigmatocystin, ochratoxin A, citrinin, penicillic acid, zearalenone and roridin A – for qualitative identification [253].

10.3 PHYCOTOXINS

10.3.1 Paralytic shellfish poisons

Toxins associated with paralytic shellfish poison (PSP) represent a group of highly polar water soluble compounds with structures as shown in Fig. 10.13. The most commonly used method at present for PSP toxin determination is the mouse bioassay [254]. It is a method that measures total toxicity of shellfish extracts and, when set up properly, is an efficient means of monitoring the safety of shellfish. However, the method requires a constant supply of mice and maintenance facilities that are not available in most analytical chemistry laboratories. As a result, research on chemical methods for PSP toxin analysis continues to be pursued.
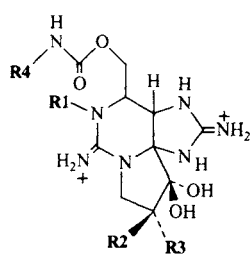
	Substituents				Toxin
	R1	R2	R3	R4	
	H	H	H	H	Saxitoxin (SAX)
	H	H	H	SO_3	B1
	H	H	OSO_3	H	Gonyautoxin 2 (GTX 2)
	H	H	OSO_3	SO_3	C1
	H	OSO_3	H	H	Gonyautoxin 3 (GTX 3)
	H	OSO_3	H	SO_3	C2
	OH	H	H	H	Neosaxitoxin (NEO)
	OH	H	H	SO_3	B2
	OH	H	OSO_3	H	Gonyautoxin 1 (GTX 1)
	OH	H	OSO_3	SO_3	C3
	OH	OSO_3	H	H	Gonyautoxin 4 (GTX 4)
	OH	OSO_3	H	SO_3	C4

Fig. 10.13. Structures of 12 PSP toxins.

10.3.1.1 Post-column oxidation reactions

The most successful of the chemical methods to date involved HPLC with post-column oxidation and fluorescence detection [255–257]. Fig. 10.14 illustrates a typical arrangement of equipment for PSP determination using the technique. The separation of the individual PSP analogues is accomplished using gradient elution ion-pair chromatography on a styrene-divinylbenzene copolymer column (PRP-1, Hamilton). The toxins are detected by fluorescence after conversion to purine derivatives by oxidation with periodate at weakly basic pH. After the oxidation, the reaction stream is adjusted to about pH 5 to optimize fluorescence yield. Fig. 10.15 shows a typical separation of a standard mixture of PSP toxins as well as chromatograms obtained with contaminated clams and mussels.

The oxidation reaction is based on earlier work [258] where PSP toxins were oxidized
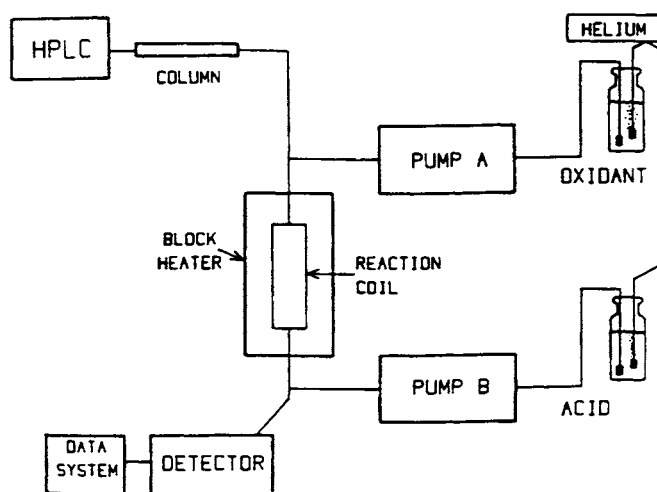
Fig. 10.14. Schematic diagram of the apparatus for postcolumn oxidation of PSP toxins. (Reproduced from Ref. [256] with permission of Elsevier, Amsterdam.)
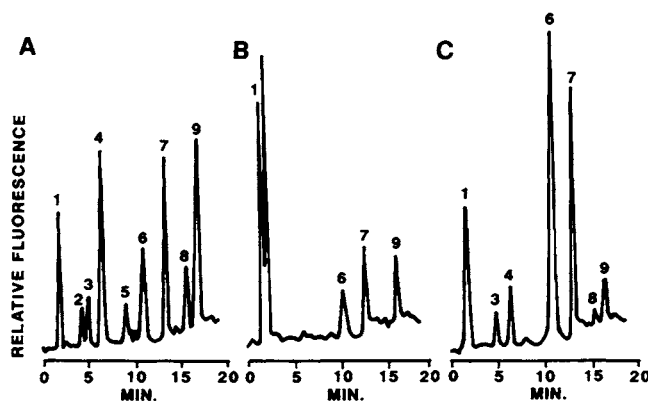
Fig. 10.15. Chromatograms of PSP toxins. 1 = Cl and C2, 2 = B2, 3 = GTX1, 5 = B1, 6 = GTX3, 7 = GTX2, 8 = neosaxitoxin, 9 = saxitoxin. (A) Toxin standards. (B) Clam extract containing 24 μg/100 g PSP. (C) Mussel extract (dilutred 1:2) containing 388 μg/100 g (Reproduced from Ref. [257] with permission of AOAC International, Arlington, VA.)

with H_2O_2 to yield fluorescent products and the total amount of fluorescence produced was used as an estimate of PSP concentration. However, since then it was found that the N-1 hydroxy-containing toxins are poorly oxidized with H_2O_2 [255], thus use of this reagent alone can seriously underestimate the true concentration of PSP in unknown extracts. Periodate was found to produce fluorescent products with all PSP products although the yields of individual analogues vary significantly. Thus, for a chemical method, it is essential to separate the analogues and quantitate them individually. Continuous flow autoanalyzers without a chromatographic separation step have been investigated [259,260]. However, these have found little use due to the problems mentioned above.

The major problem with the post-column oxidation method described above is a technological one. The system has been found to be very sensitive to slight changes in temperature, pH and chromatographic conditions, making it difficult to optimize and maintain both chromatographic separation and sensitivity for all toxins of concern. In addition, the method cannot determine the four C-toxins which, although much less toxic than saxitoxin, are of interest since they can be converted to their more toxic analogues, the gonyautoxins (GTX1–GTX4) under acidic conditions (pH 1–2) [261]. The post-column method is much more suited to monitoring for PSP contamination on an on-going basis rather than to being set up for determinations on an occasional basis.

Oshima et al. [262,263] substantially modified the post-column method developed by Sullivan and co-workers [255,256]. The significant changes made were in the chromatography, where high efficiency silica-based reversed-phase columns were employed and rather than using gradient elution, three isocratic ion-pair mobile phases were developed to determine separately the 'C' toxins, the GTX toxins, and neosaxitoxin and saxitoxin. In addition, the modified system is capable of separating and quantitating several decarbamoyl compounds (e.g. dc-saxitoxin, dc-GTX2 and dc-GTX-3) which is not possible with the gradient system employing the polymer based PRP-1 column. Also, the detection limits for individual toxins were significantly improved because of the higher efficiency
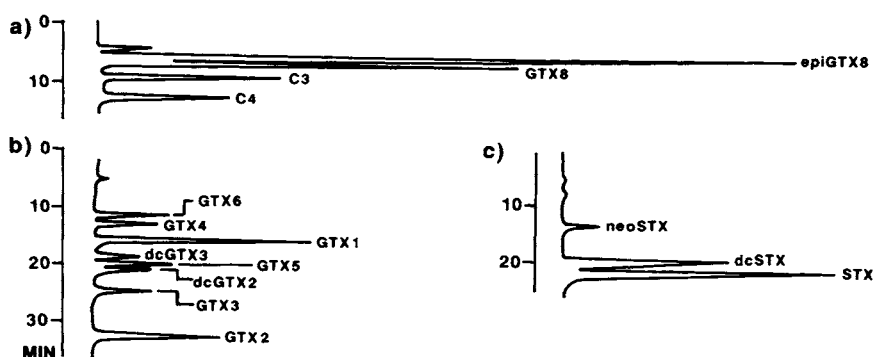
Fig. 10.16. Chromatograms of standard PSP toxins obtained using mobile phases containing (a) 1 mM tetrabutyl ammonium phosphate pH 5.0, (b) 2 mM sodium heptanesulfonate in 10 nM ammonium phosphate (pH 7.2) and (c) same as (b) but with 10% acetonitrile. epi GTX8-C1, GTX8 = C2, GTX6 = B2, GTX5 = B1. (Reproduced from Ref. [262] with permission of Elsevier, Amsterdam.

separations, compared to results obtained with the PRP-1 column. Fig. 10.16 shows typical separations obtained with this system.

Two other approaches to post column oxidation as a means of detection for PSP toxins have been described. Electrochemical oxidation, employing a coulometric cell was found to oxidize PSP toxins to the same products as with periodate oxidation [264,265]. This approach has certain advantages in that no postcolumn pumps or reagents are required making it somewhat easier to maintain. The sensitivity of the technique is similar to the standard postcolumn method; however, more work is required to determine its usefulness for routine determination of the toxins in shellfish and algal samples. A solid phase reactor consisting of a metal tube containing MnO_2 as an oxidant was evaluated for the oxidation of several PSP toxins [266]. In this approach, MnO_2 under basic conditions, oxidizes PSP toxins to fluorescent products similar to those produced by periodate. The advantage of this is that no postcolumn addition of oxidant is required; however, the addition of buffer is necessary for optimum oxidation. The method has not been evaluated for the routine analysis of PSP toxins in marine samples.

10.3.1.2 Pre-chromatographic oxidation reactions

Because of the time and special equipment required for setting up the post-column oxidation apparatus, it is not particularly well suited for laboratories in which PSP analyses are only a part of their work and not carried out on a regular basis. As a result, attempts have been made to develop a simplified HPLC method employing pre-chromatographic oxidation to form fluorescent derivatives of the individual toxins. In this approach, the oxidation reaction is carried out before the chromatography and the oxidation products are separated by HPLC and then quantitated directly with no post-column equipment. The oxidation procedure is particularly attractive as a means of derivatization since the reaction is simple, requiring only dilute periodate or peroxide at weakly basic pH. Also, the reagents themselves are non-fluorescent and thus do not interfere in the detection of the products. Luckas [267] first reported on the HPLC analysis of oxidation products of
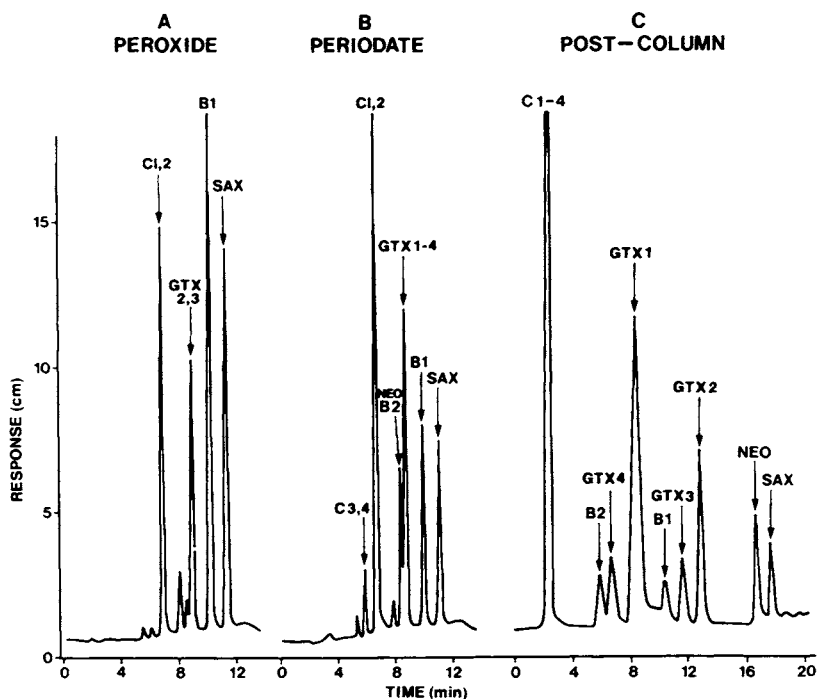
Fig. 10.17. Chromatograms of a PSP toxin standard solution. Prechromatographic oxidation (A) with peroxide and (B) with periodate. (C) Postcolumn oxidation with periodate. Quantities of saxitoxin injected: (A) 0.6 mg, (B) 0.5 ng, (C) 3.7 ng. (From Ref. [269] with permission of AOAC International, Arlington, VA).

saxitoxin after H_2O_2 oxidation. He observed a number of products and concluded that pre-column oxidation followed by HPLC would not be suitable as a reliable method for PSP determination. However, Preun et al. [268] studied the reaction and improved the chromatography so that saxitoxin produced one major peak with alkaline peroxide oxidation. This method was applied to the determination of saxitoxin in mussel samples.

    The pre-chromatographic oxidation method was studied extensively by Lawrence and co-workers [269,270] who evaluated both peroxide and periodate for the oxidation of PSP toxins under a variety of reaction conditions. They reported that the non-N-1-hydroxy toxins (saxitoxin, B1, GTX2, GTX3, C1 and C2) produced a single major peak with both peroxide and periodate oxidations. The N-1-hydroxy toxins (neosaxotoxin, B2, GTX1, GTX4, C3 and C4) did not produce fluorescent products with peroxide but did with periodate. However, all N-1-hydroxy analogues produced three fluorescent products with periodate, one of which was predominant for each toxin and could be used for quantitation. The major drawback of the method is that several of the toxins yield the same oxidation products which are not separable by reversed-phase HPLC. However, attempts at improving the quantitative aspects of the method have met with some success since a reasonable correlation with the mouse bioassay was reported [271]. Fig. 10.17 shows chromatograms of a standard mixture of toxins determined by both prechromatographic and post-column oxidation methods. The biggest advantage of the pre-chromato-
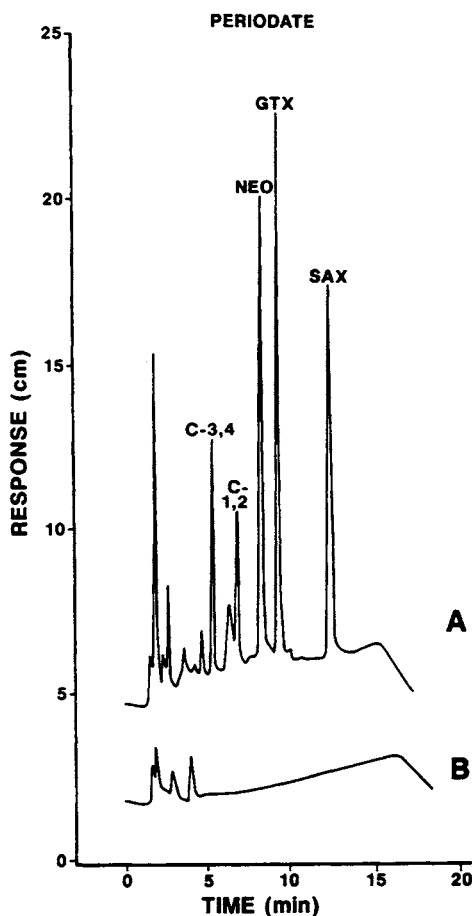
Fig. 10.18. Chromatograms of extracts of (A) contaminated (total PSP equivalent to 100 μg saxitoxin/100 g) and (B) unconditioned mussels, with periodate oxidation. (Reproduced from Ref. [270] with permission of AOAC International, Arlington, VA).

graphic oxidation method is that it employs only normal HPLC equipment (reversed-phase chromatography with fluorescence detection) and no post-column apparatus is required. In addition, the method is significantly more sensitive than the post-column method. The detection limits for individual toxins in shellfish tissue are in the low ng/g range making it much more than adequate for monitoring shellfish at regulatory levels (e.g. 80 μg/100 g, total PSP in saxitoxin equivalents). Fig. 10.18 shows typical results obtained with extracts of naturally contaminated and uncontaminated mussels. As can be seen, the presence of PSP toxins is clearly indicated in the contaminated sample.

Prechromatographic oxidation was evaluated for the detection of several decarbamoyl PSP toxins including dcStx, dcNeo, dcGTX2,3 and dcGTX1,4 [271]. It was found that two products were obtained for each dctoxin, one of which was unique to each. The unique products did not interfere with the nondecarbamoyl PSP toxin oxidation products and, thus, they could be determined in the presence of the others.

Additional work on the prechromatographic oxidation approach has been reported and includes a comparison to the mouse bioassay [272,273], a comparison with a neuroblastoma cell bioassay [274], a comparison with postcolumn oxidation and capillary electrophoresis [275] and the use of chiral chromatography to separate the oxidation products of the isomeric toxins, GTX1 and GTX4 as well as GTX2 and GTX3 [273]. Further work on the latter is required to determine its usefulness for routine determination of PSP toxins. LCMS characterization of a number of PSP oxidation products also has been reported [276] and has provided valuable information for confirmation purposes where prechromatographic oxidation is used for PSP determination in unknown samples. It was also found that the prechromatographic oxidation method can be successfully automated [277].

10.3.1.3 HPLC–MS

Several reports have appeared in the literature on the application of MS to the characterization of PSP toxins. FAB ionization MS has provided useful data on a variety of individual PSP analogues [278,279]. However, the technique is not particularly suited to determining the toxins in shellfish samples on a routine basis. The combination of HPLC–MS has been evaluated for identification of PSP toxins in shellfish. Luckas et al. [280] evaluated HPLC–TSP MS for the confirmation of decarbamoyl saxitoxin in canned mussels. They used a mobile phase of aqueous ammonium acetate containing acetonitrile to separate decarbamoyl-saxitoxin from saxitoxin. Their analysis was specifically directed at these two toxins. No application to other PSP toxins was presented.

Pleasance et al. [281] evaluated HPLC-ion spray MS for the confirmation of PSP toxins. They found that the chromatographic conditions used to separate the toxins in the post-column fluorescence method were not compatible with ion spray MS. However, they did obtain separation of GTX2, GTX3, neosaxitoxin and saxitoxin using a mobile phase containing ammonium formate and acetonitrile. Fig. 10.19 shows an example of the HPLC-ion spray MS analysis of a dinoflagellate culture containing PSP toxins. Saxitoxin, neosaxitoxin, GTX2, GTX3 and C1/2 were detected and confirmed by selected ion monitoring. Additional reports have also shown the potential of this approach [282,283] for phycotoxin confirmation and is currently the subject of extensive research by a number of groups. It should lead to some important breakthroughs in the monitoring of PSP and other marine toxins in fish and shellfish.

10.3.1.4 Other techniques

Several other methods for detection of PSP toxins have been reported, some of which are particularly useful in specific applications while others offer potential for the future. Onoue et al. [284] detected PSP toxins after HPLC separation by post-column fluorescence derivatization with OPA. This reaction, however, appears to be much less sensitive than the post-column periodate reaction described above. Also, because the OPA reagent reacts with most primary amines, amino acids and peptides, the method is much less selective, requiring a significantly greater degree of clean-up than the oxidation reactions.

One of the earliest chromatographic techniques for PSP toxins was the thin-layer chromatographic method developed by Buckley et al. [285]. PSP toxins are chromatographed on silica gel plates and after development are sprayed with 1% hydrogen peroxide solution
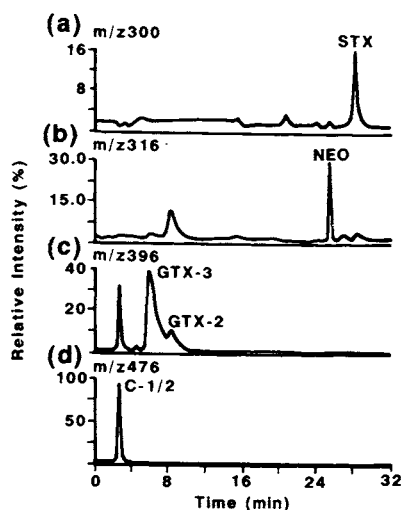
Fig. 10.19. HPLC-ion-spray MS of PSP toxins isolated from dinoflagellate (*Alexandrium excavatum*). Selected ion monitoring of MH^+ ions. Amounts injected, saxitoxin (STX) 908 ng; NEO, 247 ng; GTX2/3 141/396 ng; C1/2 797 ng. Flow rate 1 ml/min. Split = 20:1, 50 μl/min entering the mass spectrometer. PRP-1 column with mobile phase described in the text. (Reproduced from Ref. [281] with permission of Wiley, New York.)

and heated. The toxins become oxidized and are visualized under long wave-length UV light or quantitated by scanning. This method has been used successfully in research on isolation and purification of PSP toxins [286].

Electrophoresis [287] and more recently capillary electrophoresis [288–290] have been successfully employed in the separation and detection of PSP toxins. Although little work has been done on evaluating these techniques for routine screening, they do offer potential.

10.3.2 Diarrhetic shellfish poisons

Diarrhetic shellfish poison (DSP) is a term given to a group of compounds which may be present as contaminants in shellfish as a result of their consumption of certain dinoflagellates. The compounds produce severe gastrointestinal illness in humans, and as a result, there is much concern about their presence in shellfish destined for human consumption. Several countries have established regulatory guidelines limiting the presence of these substances in shellfish. The toxins associated with DSP have been determined to be large polyether compounds as shown in Fig. 10.20. They include the dinophysis toxins, the pectenotoxins and the yessotoxins [291–293]. These toxins, like most of the PSP toxins, are not readily available in sufficient quantity to enable substantial research to be carried out on chemical methods for their detection in shellfish. Because of this, the most commonly used method for DSP determination is one based on a mouse bioassay [294,295].

A number of approaches for the HPLC determination of DSP toxins have been evaluated. These mostly involve derivatization of the carboxylic acid moiety of the compounds to form highly fluorescent derivatives which are then separated by reversed-

Okadaicacid   (OA) R_1=H, R_2=Me, R_3=H
Dinophysistoxin-1 (DTX1) R_1=H, R_2=Me, R_3=Me
Dinophysistoxin-2 (DTX2) R_1=H, R_2=H, R_3=Me
Dinophysistoxin-3 (DTX3) R_1=acyl, R_2=Me, R_3=Me

Pectenotoxin-1 (PTX1)   R=CH_2OH
Pectenotoxin-2 (PTX2)   R=CH_3
Pectenotoxin-3 (PTX3)   R=CHO
Pectenotoxin-6 (PTX6)   R=COOH

yessotoxin (YTX)
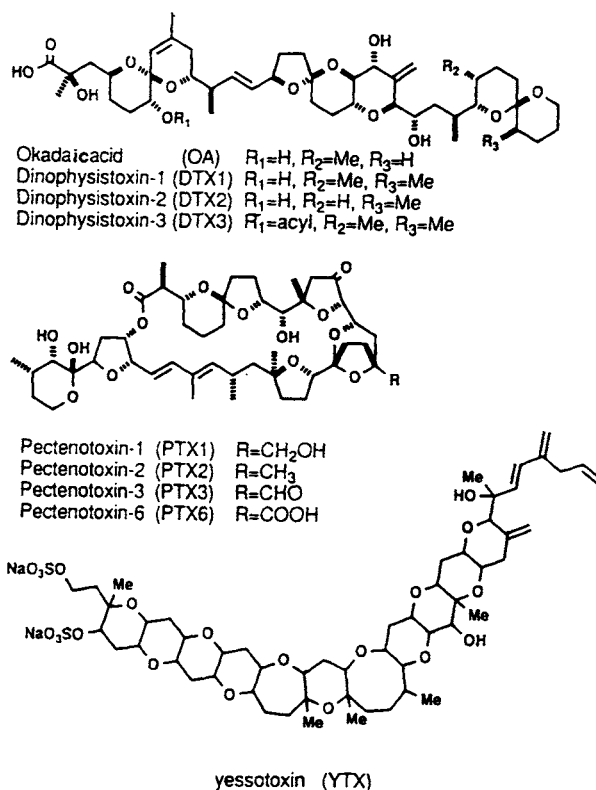
Fig. 10.20. Structures of DSP toxins.

phase chromatography. The method which has received most attention up to the present is that developed by Lee et al. [296] for the determination of okadaic acid and DTX-1. The method involves extraction of the samples with 80% MeOH in H_2O followed by partitioning with petroleum ether and chloroform. The chloroform extract is then derivatized with 9-anthryldiazomethane (ADAM) to yield highly fluorescent products shown in Fig. 10.21. After derivatization, the reaction mixture is further cleaned up by passage of the solution through a disposable silica gel SPE cartridge. Fig. 10.22 shows typical chromatograms obtained for okadaic acid and DTX-1 added to an extract of mussel digestive glands. As

9-anthryldiazo-
methane (ADAM)
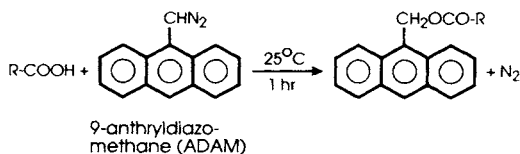
Fig. 10.21. Reaction of 9-anthryldiazomethane with carboxylic acids to form flourescent derivatives.
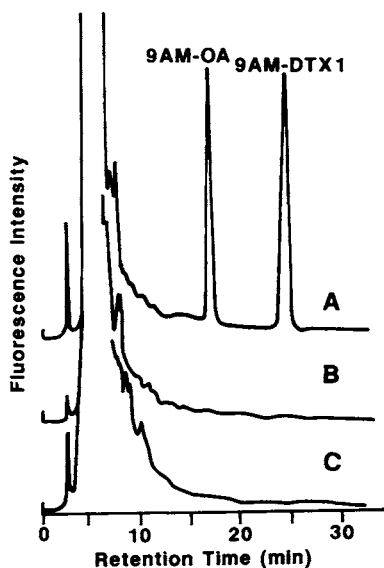
Fig. 10.22. Chromatograms of (A) 9-anthryldiazomethane esters of okadic acid (3 μg/g) (9AM-0A) and DTX-1 (6 μg/g) (9AM-DTX1) added to an extract of non-toxic mussel digestive glands. (B) Blank non-toxic mussel digestive glands. (C) Extract of non-toxic scallop digestive glands. (Reproduced from Ref. [296] with permission of the Agric. Chem. Soc. Japan, Tokyo.)

can be seen, both okadaic acid (3 μg/g) and DTX-1 (6 μg/g) are readily detected at these spiking levels.

A number of modifications of the method of Lee et al. [296] have been reported. An improved clean-up procedure [297] involving a modification of the silica gel clean-up and the use of an internal standard (deoxycholic acid) to monitor the efficiency of the ADAM derivatization reaction enabled the detection of levels as low as 40 ng/g of okadaic acid or DTX-1. A column switching technique which avoided the use of the silica gel clean-up has also been described [298]. In this method, derivatized extracts are injected into the HPLC (reversed-phase) system. The okadaic acid and DTX-1 derivative peaks are 'heart-cut' and directed onto an enrichment column after which they are switched again to a third column where they are separated and detected fluorometrically. The method appears somewhat complex requiring three columns and switching valves. It has not been evaluated extensively on a routine basis. The influence of the extraction procedure [299] and the sample clean-up [300] on recovery of OA from shellfish for determination by both HPLC and bioassay has been studied. A comprehensive evaluation of the ADAM method was carried out by Quilliam [301] for both OA and DTX-1. Since the ADAM reagent is relatively expensive and of limited stability, a method for synthesizing it immediately before use has been described [302,303]. This approach gives comparable results to that obtained using purified ADAM.

Some investigators have evaluated a number of coumarin derivatives as suitable replacements for ADAM. The reagent, 4-bromomethyl-7-methoxy coumarin, was found to yield highly fluorescent derivatives with both okadaic acid and DTX-1 [304]. This reagent is one of a series of coumarin compounds that have been evaluated for forming fluorescent
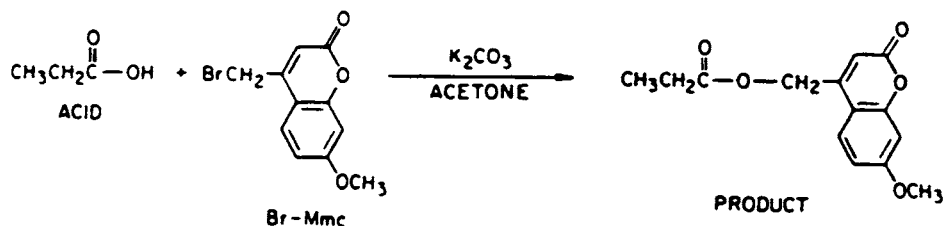
Fig. 10.23. Reaction of 4-bromomethyl-6-methoxycoumarin with carboxylic acids.

derivatives of carboxylic acids [305]. Several have been compared for application to DSP determination and it was found that 4-bromomethyl-6,7-dimethoxy coumarin produced the most sensitive derivative [306]. Fig. 10.23 shows a generalized reaction scheme. Although the coumarin type reagents are stable and reasonably priced, they appear to be somewhat less selective than ADAM, necessitating additional sample clean-up before HPLC determination. However, they do offer potential for routine determinations.

The reagent, 1-bromoacetylpyrene (BAP), was examined and found to be useful for OA, DTX-1 and DTX-2 [307,308]. Although the derivatives were about 4 times less fluorescent than the ADAM products, the chromatographic properties were similar. BAP was found to be more stable than ADAM and yielded more consistent chromatograms. Another reagent which has been evaluated for okadaic acid and DTX-1 is N-(9 acridinyl)-bromoacetamide [303,309]. The reaction involved phase-transfer catalyzed esterification in an aqueous/organic two-phase system. As little as 10 fmol of carboxylic acid can be detected with the procedure. The compound 9-chloromethylanthracene (CA) was also examined for use as a derivatization reagent for OA and DTX-1 [310,311]. This reagent provides the same products as the ADAM reaction but has the advantage that it is stable at room temperature and relatively inexpensive. The results using this reagent appear to be similar to those obtained using ADAM, although a longer reaction time and higher temperature are required for the CA reaction.

The major problem with all of the above methods is that okadaic acid and DTX-1 are very similar in polarity to many naturally occurring fatty acids. In order for any of these derivatization reactions to work, it is necessary to remove as much as possible potentially interfering carboxylic acids. Further work on the selective clean-up of these toxins should enable their detection limits to be significantly lowered. However, there always remains the trade-off of increased sample clean-up and improved detection limits with analysis time and method reproducibility due to increased sample handling.

There have been a number of reports on the direct determination of DSP toxins in shellfish that have involved HPLC combined with mass spectrometry [303,312–316]. With this technique, extracts of shellfish can be analyzed directly without derivatization and with little or no clean-up of the extract. Fig. 10.24 compares HPLC–MS results with those obtained with the HPLC-ADAM method for OA and DTX-1. A good agreement was found between the two methods. Although the HPLC–MS equipment is somewhat too expensive for most routine analytical laboratories, this work shows that the approach offers excellent potential for confirming positive findings by the HPLC-ADAM or other technique. For research purposes, for identifying new toxins, the approach is the best available. HPLC–MS has already led to the discovery of new DSP toxins [312–315].
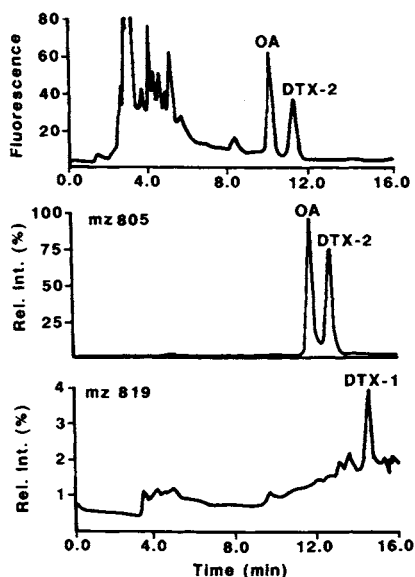
Fig. 10.24. Comparison of HPLC-ADAM (a) with HPLC-ion-spray MS (b,c) for okadaic acid (OA), DTX-1 and DTX-2 in contaminated Irish mussels. (Reproduced from Ref. [312] with permission of Wiley, New York.)

Micellar electrokinetic chromatography (MECK) using UV absorbance detection has been applied to the direct determination of OA and DTX-2 in shellfish and crude dinoflagellate extracts [317]. Although detection of OA at 10 ng/g whole tissue was accomplished, the quantitative aspects still require improvement.

Very little analytical method development work has been reported for the pectenotoxins or the yessotoxins, due primarily to the lack of analytical standards. A fluorometric labeling method for HPLC analysis has been reported for the pectenotoxins [318]. The method involves reaction of the toxins with 1-anthroyl nitrile which reacts with primary hydroxyl groups [319]. It has been applied to the determination of PTX-1 and PTX-4 in scallops and to PTX-2 in *Dinophysis fortii* with FAB MS confirmation.

The same authors [318] reported on a direct HPLC (reversed-phase) method for the detection of YTX and 45-hydroxy-YTX in Norwegian and Japanese mussels. The method made use of the natural UV absorption of the compounds at 230 nm. More work needs to be carried out on these methods to determine accuracy, reproducibility and detection limits which are required before they can be employed for regulatory purposes.

The reagent DMEQ-TAD, a dienophile, has been reported for the determination of yessotoxin and related toxins [320]. It reacts with the compounds to form fluorescent derivatives. Further evaluation of this reagent is required for routine application to shellfish and dinoflagellate samples.

10.3.3 Domoic acid

Although domoic acid (structure, Fig. 10.25) was first isolated in 1958 from the red alga
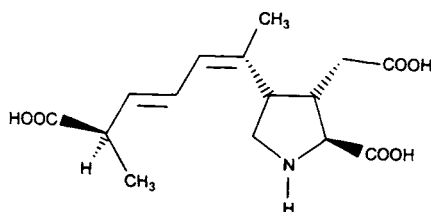
Fig. 10.25. Structure of domoic acid.

Chondria armata [321], it was not until an outbreak of domoic acid poisoning in humans in 1987 [322] that research into method development was carried out to any great extent. The first analytical method for determining domoic acid in shellfish involved reversed-phase HPLC analysis with UV detection of the underivatized compound at its absorption maximum of 242 nm [323]. Since then several other methods have been reported, differing mainly in extraction procedure [324–326]. These methods either employed boiling water, boiling 0.1 N HCl or methanol/water as extracting solutions. The last approach appears to be in most common use at present as it does not require heating. The acid extraction procedure is actually the same one that is used for the PSP mouse bioassay. The use of it is convenient if both PSP and domoic acid are to be determined on the same samples. It has
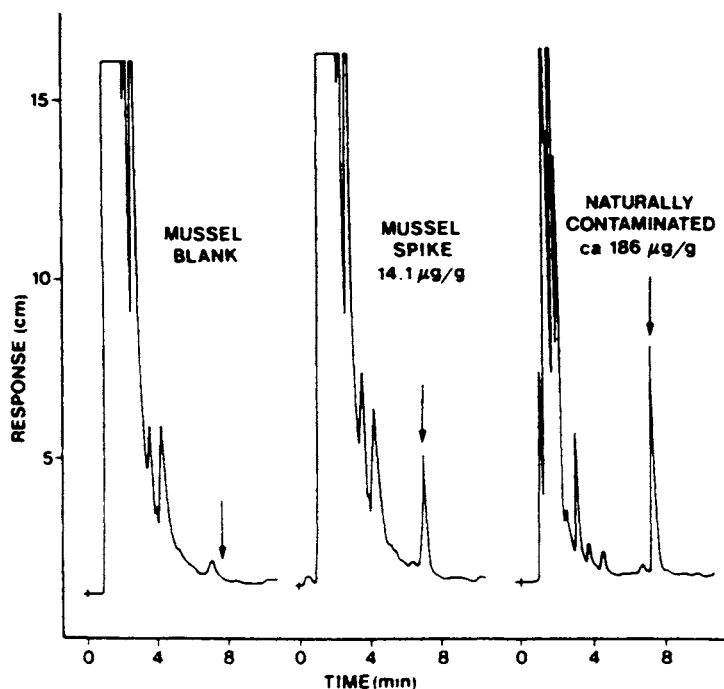
Fig. 10.26. Chromatograms of a blank mussel extract, a blank mussel extract plus 14.1 μg/g domoic acid and a naturally contaminated mussel extract. Arrow indicates domoic acid retention time. (Reproduced from Ref. [327] with permission of AOAC International, Arlington, VA.)

been successfully collaboratively studied by AOAC International [327]. Fig. 10.26 shows typical chromatograms of domoic acid in mussel tissue using the collaboratively studied method. However, recoveries were only about 70–75% and the extracts were not stable enough to store for more than a few days. The methanol/water extraction procedure [326] yields cleaner and much more stable extracts. The method is presently being evaluated through an interlaboratory collaborative study.

The methanol/water extraction method [326] was evaluated in a comparison study with both an immunoassay and an HPLC-mass spectrometric method [328,329]. A very good correlation among the methods was obtained and showed that the three approaches can act as confirmatory tests for each other. Column-switching has been evaluated as a means of obtaining clean extracts for domoic acid quantitation in shellfish samples [330,331].

Several chemical derivatization methods have been investigated either to confirm results obtained by direct HPLC [332,333] or for detection of extremely low amounts of domoic acid in, for example, sea water [334]. A confirmation procedure need only be as sensitive as the original analytical procedure. Thus the main aim of chemical derivatization in this case is to create a derivative of domoic acid with a different retention time. If, in an unknown sample, a peak is observed at the same retention time and with a similar UV spectrum as domoic acid, then derivatization of that substance should yield the same product as domoic acid, if, indeed, the unknown peak was domoic acid. This gives additional information in identifying domoic acid in unknown samples. Several reactions have been evaluated for this. Esterification of the carboxylic acid groups with hydrochloric acid and various alcohols led to the formation of domoic acid triesters [332]. The advantage of this reaction is that the reagents do not contain strongly absorbing chromophores which may yield UV absorbing derivatives with sample co-extractives. However, in spite of this, additional sample clean-up was necessary because sample co-extractives in the shellfish samples interfered by consuming the derivatization reagent, resulting in low esterification yields for domoic acid.

Several N–H reactive reagents have also been evaluated. Phenyl- and butyl-isothiocyanate were evaluated for confirmation using UV detection [332,333]. Phenyl-isothiocyanate, used for amino acid analyses, proved to be somewhat less satisfactory than the butyl reagent since the former produced UV-absorbing derivatives with much of the co-extracted material in the sample extracts that led to interferences in detecting the domoic acid derivative [332]. Additional clean-up of the extracts with SPE cartridges was required. The butyl-isothiocyanate reagent [333] was more successful but it too required additional sample clean-up when the domoic acid levels were in the low μg/g range. Fig. 10.27 illustrates the effect of cleaning up a spiked mussel extract on the derivatization of domoic acid. The SPE treatment (strong cation exchange cartridge followed by a reversed-phase C-18 cartridge) provided very good clean-up enabling the confirmation of domoic acid at low μg/g levels.

Only one report has appeared on the fluorescence derivatization of domoic acid [334]. The reagent employed was 9-fluorenylmethyl chloroformate (FMOC). It reacts with the N–H moiety of domoic acid to form a highly fluorescent derivative detectable in sub-nanogram quantities. The method was applied to the detection of 15 pg/ml levels of domoic acid in sea water. This reaction also required additional sample clean-up. A post-column HPLC method for domoic acid using the ninhydrin reaction was reported [335]. A variety of shellfish were tested and the method found to compare very well with
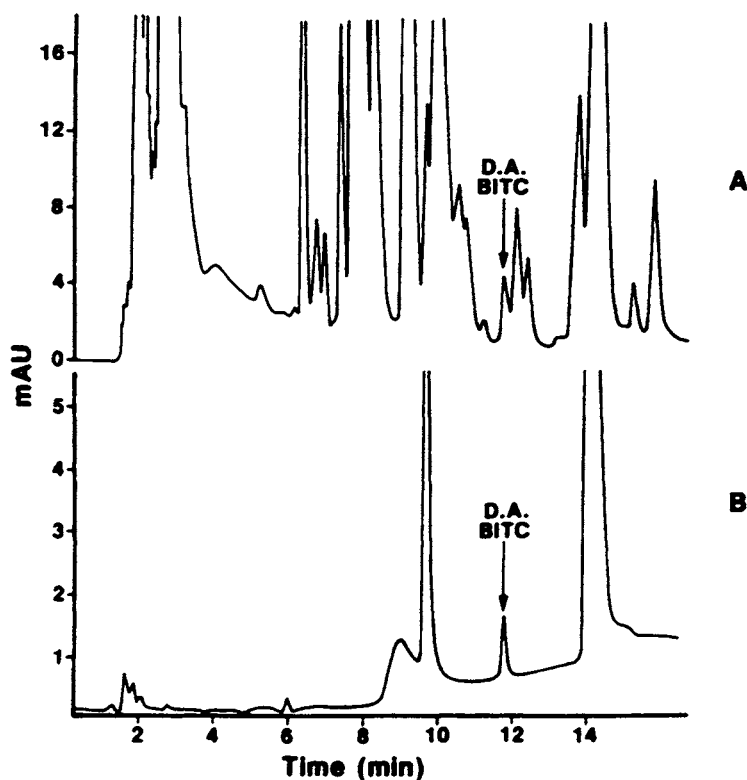
Fig. 10.27. Chromatograms of uncleaned (A) and cleaned (B) extracts of domoic acid (DA) spiked (22 μg/g) mussels after butylisothiocyanate (BITC) reaction.

the direct HPLC–UV method. The authors suggested that the post-column technique could serve as a confirmatory tool for domoic acid.

HPLC combined with ion-spray MS has been shown to be particularly useful for confirmation of domoic acid in shellfish [336]. Fig. 10.28 shows a selected ion chromatogram of an extract of domoic acid contaminated mussel tissue (containing 37 μg/ml in the
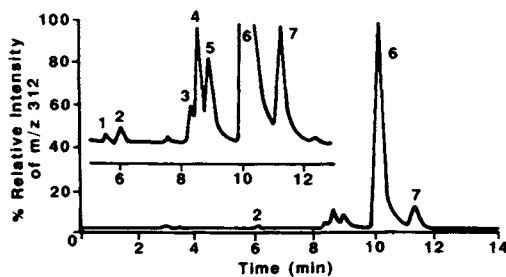
Fig. 10.28. Ion-spray HPLC/MS chromatogram of a toxic mussel extract (37 μg/ml domoic acid, peak 6). Other numbered peaks are domoic acid isomers. Positive ion mode, selected ion monitoring of m/z 312. (Reproduced from Ref. [336] with permission of Wiley, New York.)

extract). As can be seen, domoic acid (peak 6) is readily detected at this level. In addition, all other numbered peaks have been identified as domoic acid isomers. Research on the interrelationship of the isomers, their toxicity and stability is still required.

Capillary electrophoresis, a technique of much interest in analytical chemistry at present, has been investigated for the detection and quantitation of domoic acid [337,338]. The method employs UV absorbance detection and could detect as low as 3 μg/g domoic acid in mussel tissue.

Overall, the HPLC methodology for domoic acid is more than adequate for monitoring at the suggested Canadian guideline level of 20 μg/g. The direct HPLC–UV method requires minimal clean-up and employs HPLC equipment routinely used in most trace analytical laboratories.

10.3.4 Other phycotoxins

Tetrodotoxin and its derivatives (Fig. 10.29) are non-proteinaceous toxins found in a variety of fish most notably the pufferfish [339,340]. Nine tetrodotoxin analogues have now been identified [341,342]. Methods for analysis of tetrodotoxin have involved HPLC with post-column reaction with NaOH [343–345] or OPA [346]. The latter reaction is much less selective since it gives fluorescent products with any primary amine co-extractives in the sample extract. The post-column method employing NaOH yields cleaner chromatograms and could detect low nanogram quantities of tetrodotoxin which enabled the determination of less than 1 μg/g of the toxins in fish tissue.

Other phycotoxins of concern are the brevetoxins (produced by the marine dinoflagellate *Ptychodiscus brevis*), ciguatoxin (from *Gambierdiscus toxicus*) and the freshwater toxins, anatoxins, aphantoxins and microcystins (produced by cyanobacteria). Analytical methods for these compounds are still very much in the developmental stages due mainly to the lack of pure standards for research purposes. HPLC has been used in the purification of brevetoxins [347], ciguatoxins [348] and anatoxins [349].An HPLC method for ciguatoxins has been reported that makes use of the formation of a fluorescent derivative with 1-anthroylnitrile[350]. HPLC–MS methods have also been reported for this class of toxins
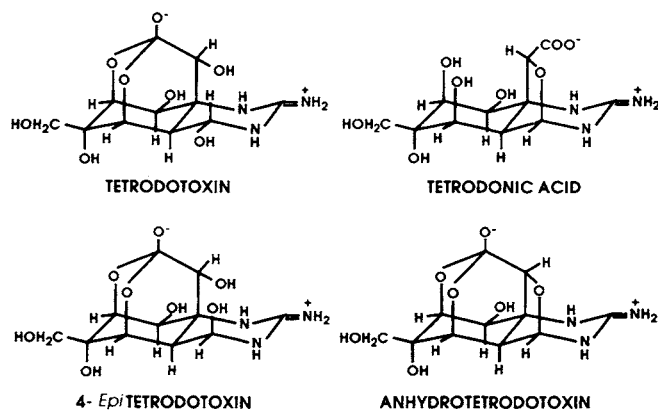
Fig. 10.29. Structures of tetrodotoxin and three derivatives, tetrodonic acid, 4-epitetrodotoxin and anhydrotetrodoxin.

[351–353]. Capillary electrophoresis was evaluated for the detection of maitotoxin[354]; however, further evaluation of this approach is required.

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Chapter 11

Determination of radionuclides in environmental samples

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CONTENTS

11.1 INTRODUCTION

The determination of radionuclides in environmental samples is important in relation to the protection of human health. This is especially the case when there is an accidental release of radioactivity into the environment, as was the case with the Chernobyl accident. The assessment of such a situation requires knowledge of the type and form of the released radionuclides, and reliable and practical techniques for the analysis of the various radionuclides.

Numerous sources of ionizing radiation can lead to human exposure: natural sources, nuclear explosions, nuclear power generation, the use of radiation in medical industrial and research purposes, and radiation-emitting consumer products. Before assessing the radiation dose to the population one requires a precise knowledge of the activity of a number of radionuclides. The measurement of radioactive contamination is the basis for the assessment of any dose to the population from a release of radioactivity into the environment, the estimation of the potential clinical health effects from to the dose received and, ultimately, the implementation of countermeasures to protect the population.

The two basic quantities for the assessment of radiation levels and their effects are the activity of a radioactive material and the radiation dose. The activity of a radioactive material is the number of nuclear disintegrations per unit time; the unit being becquerels (Bq). One becquerel is one disintegration per second. The term radiation dose can mean several things (e.g., absorbed dose, dose equivalent or effective dose equivalent). The absorbed dose of radiation is the energy imparted per unit mass of the irradiated material. The unit of absorbed dose is joule/kg, for which the special name gray (Gy) is used.

The purpose of this review is to summarize the main aspects of the problem of determination of radionuclides in the environment.

11.2 RADIOACTIVE NUCLIDES IN NATURE

Except for the simplest nucleus, that of hydrogen, all other nuclei consist of neutrons and protons. The ratio of neutrons to protons is unity for the lighter isotopes on the so-called 'stability line' and increases gradually as one approaches the behaviour of elements at the end of the periodic table or moves off the 'stability line'. As this ratio increases, a stage is reached where the nuclide is no longer stable. The heaviest stable nuclide is ^{209}Bi. Nuclides heavier than this are unstable because they have excess energy to dissipate. Unstable nuclides are called radionuclides, and they dissipate their surplus energy by the emission of radiation. This process is called radioactivity or radioactive decay.

Radioactivity can be of two types: (1) natural radioactivity exhibited by more than 50 naturally occurring isotopes (e.g., ^{238}U, ^{226}Ra, ^{40}K), and (2) artificial radioactivity which is the radioactivity induced in some elements by bombarding them with neutrons, charged particles or photons. The resultant nuclei (e.g., ^{137}Cs, ^{60}Co, ^{32}P) could be in excited states and will therefore decay by one of the modes described above.

Radionuclides which can be found in the environment can be divided into three groups:

- Naturally occurring nuclides of very long half-life which have persisted since the formation of the Earth, and their shorter-lived daughter nuclides

- Naturally occurring nuclides which have short half-lives on the geological time scale, but which are being continuously produced by cosmic-ray radiation
- Radionuclides released into the environment owing to man's activity and accidents

Some of the long-lived naturally occurring radionuclides are shown in Table 11.2. Some elements in this table result in non-negligible doses to man. For example, potassium, which is an essential element, is under close homeostatic control in the body. The average mass-concentration for an adult male is about 2,g of potassium per kg of body weight. The isotopic ratio of ^{40}K is 1.18×10^{-1}, and the average activity mass concentration of ^{40}K in the body is about 60 Bq kg^{-1}.

Other nuclides in Table 11.1 which are of particular interest are uranium and thorium isotopes and their series. Number of radionuclides is formed during their decays. As an the illustration, Table 11.2 shows the masses of the various daughter nuclei in secular equilibrium with l g of ^{238}U. Of special interest are the gases radon (^{222}Rn) and 'thoron' (^{220}Rn) which are formed as progeny of uranium and thorium in rocks and soil. They are emitted from the ground into the atmosphere, where they decay and form daughter products – isotopes of polonium, bismuth and lead – which either remain airborne till they decay, or are deposited by rain and by diffusion to the ground.

The second group includes radioisotopes produced by cosmic rays. The rates of production of radioactive isotopes can be estimated reasonably well from the energy spectra of primary and secondary cosmic rays and a knowledge of the corresponding nuclear reaction cross-sections.

During the passage through the atmosphere, a number of nuclear reactions takes place,

TABLE 11.1

SOME NATURAL RADIONUCLIDES WITH LONG HALF-LIVES

Radionuclide	Half-life (10^9 years)	Specific activity/Ci (g of element)$^{-1}$	Radioactivity
^{40}K	1.27	8.3×10^{-10}	β
^{50}V	6×10^5	2.8×10^{-14}	β
^{87}Rb	47	2.5×10^{-8}	β
^{115}In	6×10^5	5.0×10^{-12}	β
^{138}La	110	2.1×10^{-12}	β
^{142}Ce	6×10^6	5.7×10^{-14}	α
^{147}Sm	110	3.4×10^{-9}	α
^{148}Sm	1.2×10^4	2.2×10^{-11}	α
^{149}Sm	4×10^5	8.2×10^{-13}	α
^{152}Gd	1.1×10^5	4.1×10^{-12}	α
^{174}Hf	4.3×10^6	8.4×10^{-14}	α
^{144}Nd	5×10^6	1.2×10^{-13}	α
^{190}Pt	700	3.3×10^{-13}	α
^{192}Pt	10^6	1.4×10^{-14}	α
^{204}Pb	1.4×10^8	1.8×10^{-16}	α
^{232}Th	14	1.1×10^{-7}	α
^{235}U	0.71	1.5×10^{-8}	α
^{238}U	4.5	3.3×10^{-7}	α

TABLE 11.2

MASSES OF THE VARIOUS DAUGHTERS IN SECULAR EQUILIBRIUM WITH 1 g OF ^{238}U

Isotope	Mass (g)
^{238}U	1.0
^{234}Th	1.4×10^{-11}
^{234m}Pa	4.8×10^{-16}
^{234}U	5.4×10^{-6}
^{230}Th	1.8×10^{-5}
^{226}Ra	3.3×10^{-7}
^{222}Rn	2.2×10^{-10}
^{218}Po	1.2×10^{-15}
^{214}Pb	1.0×10^{-14}
^{214}Bi	7.4×10^{-15}
^{212}Pb	4.1×10^{-9}
^{210}Bi	2.7×10^{-12}
^{210}Po	7.4×10^{-11}

and these are responsible for the production of a wide variety of radionuclides (see Table 11.3).

The greatest part of the external gamma dose-rate above typical soils (95%) arises from primordial radionuclides trapped in the soil.

The main contributors to this component are as follows:

^{40}K   35%
^{232}Th series   50%
^{238}U series   15%

The main contributors to the gamma dose-rate in terms of specific radionuclides are ^{208}Tl and ^{228}Ac from the ^{232}Th decay chain, while for the ^{238}U decay-series about 99% of the dose-rate is from ^{214}Pb and ^{214}Bi. Gamma energies range up to 2.6 MeV and are partly attenuated by the soil with the result that for a typical exposure situation above ground the predominant contribution to the gamma dose-rate arises from radioactive material in the top 30 cm of soil.

Typical world averages and ranges in normal soils (wet weight) for uranium and thorium are:

^{238}U   0.025 Bq g^{-1} (0.01–0.05)
^{232}Th   0.025 Bq g^{-1} (0.07–0.05)

One should note the values higher by orders of magnitude can occur in soils in specific localized areas of the world. There are two major factors accounting for this variability: radionuclide concentrations and shielding.

The dose-rates will vary according to the geology of an area and the radionuclide composition of the rocks and soil. The highest levels are generally found in igneous rocks, which is this is related to the fact of quantity of silicates is highest in acidic rocks. In general, sedimentary rocks have lower concentrations of radioactive materials than igneous rocks although some shales and phosphate rocks can exhibit significantly elevated levels. The radioactivity in soils is primarily that of the rock from which it was derived, but this can be diminished or augmented by weathering, sedimentation, leaching/

TABLE 11.3

HALF-LIVES AND DECAY CHARACTERISTICS OF COSMIC-RAY PRODUCED RADIONUCLIDES

Radionuclide	Half-life	Main radiation
^{10}Be	1.6×10^6 years	β 555 keV
^{26}Al	7.2×10^5 years	β^+ 1.17 MeV; γ 1.81 MeV, 511 keV
^{36}Cl	3.00×10^5 years	β 714 keV
^{80}Kr	2.13×10^5 years	K-X-ray
^{14}C	5730 years	β 156 keV
^{32}Si	ca. 650 years	β 210 keV
^{39}Ar	269 years	β 565 keV
^{3}H	12.33 years	β 18.6 keV
^{22}Na	2.60 years	β^+ 0.545, 1.82 MeV; γ 1.275 MeV, 511 keV
^{36}S	87.4 days	β 167 keV
^{7}Be	53.3 days	E.C., γ 477 keV
^{37}Ar	35.0 days	K-X-ray, Bremsstrahlung to 0.81 MeV
^{33}P	25.3 days	β 248 keV
^{32}P	14.28 days	β 1.710 MeV
^{28}Mg	21.0 h	β 0.459, γ 1.35, 0.31, 0.95, 0.40 MeV
^{24}Na	15.02 h	β 1.389 MeV; γ 1.369, 2.754 MeV
^{38}S	2.83 h	β 3.0, γ 1.88 MeV; γ 1.6, 2.17 MeV
^{31}Si	2.62 h	β 1.48 MeV; γ 1.26 MeV
^{16}F	109.8 min	β^+ 0.635 MeV; 511 keV
^{39}Cl	56.2 min	β 1.91 to 3.45 MeV; γ 0.246, 1.27, 1.52 MeV
^{38}Cl	37.29 min	β^+ 4.91 MeV; γ 1.6, 2.17 MeV
^{34m}Cl	31.99 min	β^+ 2.48 MeV; e^- 0.142 MeV; γ 1.17, 2.12, 3.30 MeV; 511 keV

sorption, and precipitation from moving groundwater, dilution with other materials, and increased porosity.

Shielding is another factor to be considered. For example, 20 cm of snow cover will reduce the gamma dose-rate by half. During wet weather, attenuation to water-logging in the topsoil can reduce local gamma dose-rates by up to 20%.

There are locations where the terrestrial radiation is locally elevated, owing mainly to the uranium and thorium mineralization.

Rivers erode soils which contain radionuclides, which then reach lakes and oceans. Atmospheric depositions can also land on their surfaces. Groundwater with contains some radionuclides can leach onto them. Therefore, water contains a small and variable quantity of natural radioactivity from the decay of uranium and thorium and their daughters, together with ^{40}K. The background radiation has increased during the past three decades as a result of man's exploitation of nuclear fission. The original, and still the major, artificial input to the hydrosphere is from the fallout of fission products from nuclear weapons testing. The presence, in rain- and river water, of ^{90}Sr and ^{137}Cs (and other radionuclides) has been well documented [1,2]. Other sources of radioactivity now include the discharge of small quantities of liquid radioactive waster from the operation of nuclear-powered electricity generating stations and research establishments, the use of

radioactive materials in industry and medicine, and also from the use of tracers for the investigation of water- and sediment movement.

The control of the uses of radioactivity and the limitation of discharges to the environment should ensure that the levels in water are below the limits derived from the International Commission on Radiological Protection's (ICRP's) recommendations. Where appropriate, the radioactive content of water is measured by the operator who is authorized to discharge the radioactivity, and the results are checked by the appropriate authorizing Government Departments. In addition, tracer experiments to follow water movement are usually carried out by specialist groups with the appropriate measuring equipment. The measurement of the radioactive content of water is carried out by some governments' Water Authorities as a check on trends and natural levels to be expected in the environment.

The concentration of atmospheric radionuclides have special distributions which depend on latitudes and altitudes. Cosmogenic radionuclides have higher production rates in the stratosphere than in the troposphere, because of higher intensity of cosmic rays in the stratosphere. Fallout nuclides have higher concentrations in the mid-latitudes of the northern hemisphere, because most atmospheric nuclear explosion experiments were made there.

Potassium is an essential element that is under close homeostatic control in the human body. The radionuclide is both a beta and gamma-emitter: consequently the whole body is uniformly irradiated. ^{40}K is the principal naturally occurring source of internal radiation arising from ingestion. Stable potassium enters the body mainly via foodstuffs, at the rate of about 2.5 g day^{-1}. Specific locations in the body where potassium is preferentially concentrated (such as the bone narrow) receive the highest doses.

Radon and thoron and their decay products are the most important sources of radiation exposure to the general public, contributing on average about half of the total effective dose-equivalent received from natural and man-made radioactivity [3–5]; see Table 11.4.

Radon (^{222}Rn or Rn) is a radioactive noble gas, which is chemically relatively inert. The gas originates from traces or uranium (^{238}U or U) in various materials, e.g., granites. The Rn atom is formed in the mineral grains of the material. A certain fraction of atoms formed

TABLE 11.4

AVERAGE INDIVIDUAL RADIATION EXPOSURES FROM VARIOUS SOURCES

Radiation sources	Effective dose equivalent year^{-1} m^{-1} Sv
Natural	
Cosmic rays at sea level	0.37
Radon (^{222}Rn and ^{220}Rn)	1.30
Potassium (^{40}K)	0.30
Other natural sources	0.40
Man-made	
Medical use of radiation	0.4–1.0
Nuclear explosives testing	0.01
Nuclear power production	0.002

escapes into the material pore-space and diffuses to the material surface to be released into the surrounding air. Radon decays radioactively into a series of decay products, or Rn daughters (RnD), which are themselves radioactive. Inhalation and subsequent deposition in the respiratory tract of these RnD constitutes a radiation hazard, owing to the irradiation of the respiratory tract tissue during their decay. The fact that Rn is present in all soils, and is measurable down to very low concentrations, makes it an ideal, naturally occurring tracer gas. This property may be used, e.g., in exploration techniques, or in the detection of subsoil fissures and aquifers.

A Rn atom is formed upon decay of the predecessor ^{226}Ra atom in the mineral grains of the U-bearing material. When the decay happens close to the grain surface, the Rn atom can, by virtue of its recoil energy, be ejected from the mineral grain into the material's pore space. The moisture content of the material has an effect on this process, as a thin moisture layer in the grain surface can retard the recoiling Rn atom so that it ends in the pore space. If no such layer exists, the atom may be ejected into an adjacent grain, and not be available in the pore space. If the pores are filled with water, the atom ends in the water layer. It follows that only a certain fraction of all the Rn atoms formed in the material grains can escape into the pore space. This is the 'emanation fraction' of the material. In the pore space the Rn atom diffuses through the tortuous pore paths. This process is governed largely by diffusion, although convective processes may also assist in the transport, e.g., if pressure differentials exist across the material. The Water content again affects the transport to a very large extent, since the diffusion of Rn in water is about 100 times slower than in air.

There can be large short-term localized variations around an 'average' Rn concentration, directly related to atmospheric stability conditions. Strong inversion layers close to the ground can act to trap radon and daughters and result in increases in the gamma dose-rate up to 25% or more. Similar temporary increases can occur in heavy rainstorms when the radon daughters are washed out onto the ground surface.

^{222}Rn concentrations in outdoor air are largely dependent on the ^{226}Ra concentration in the soil, the exhalation rate from the soil, and atmospheric dispersion factors. Values over large areas of water will be lower than over land areas, and values near surface uranium deposits can be up to several orders of magnitude higher. Diurnal and seasonal variations are of the order of 2.4 times the lowest values.

Indoor concentrations are more variable especially in northern climates where low ventilation rates are encountered inside sealed and insulated buildings. As a result, the greatest past of the exposure arises indoors, and may be many times the outdoor contribution.

11.3 TECHNOLOGICALLY MODIFIED EXPOSURE TO NATURAL RADIATION

There are circumstances where man finds himself in a natural radiation environment to which he would not be exposed if some kind of technology had not been developed. Examples are travelling by air, using natural gas for cooking or heating purposes, or living in the neighbourhood of a coal-fired power plant. The resulting exposures have been labelled 'technologically enhanced' natural radiation exposures. They are defined as

exposures to truly natural sources of radiation (that is, naturally-occurring radionuclides and cosmic radiation) which would not occur without (or which are increased by) some technological activity.

In some cases, technology helps to reduce the natural radiation exposure. For example, when drinking-water supplies are drawn from surface waters, the use of water-purification processes brings about a reduction in the concentration of radium and other naturally occurring radioactive elements. Another example is the burning of fossil fuels, which reduces the specific activity of ^{14}C in the biosphere and therefore reduces the doses from that of radionuclides.

Operations and activities which act to concentrate and redistribute naturally occurring radioactive material in the environment are numerous. For example coal, like most materials found in nature, contains trace quantities of the naturally occurring primordial radionuclides. Therefore, the combustion of coal results in the release to the environment of some natural activity and in the re-distribution of that natural activity from deep in the earth to locations where it can modify ambient radiation fields and population radiation exposure.

During recent years, radioactivity released by coal power stations into the environment has received great attention from the public, as well as from governments and their agencies. Some of the reports have received much attention, and have been published in journals and newspapers. One of these reports, much discussed by the general public, is derived from the study conducted by the US Environmental Protection Agency (EPA). The report, 'Radiological Impact Caused by Emission of Radionuclides into Air in the United States', was prepared as a result of legislation that requires radionuclides to be included among other sources of air pollution. Preliminary findings suggest that there are greater risks to the public of developing cancer from radionuclides emitted by coal-fired power plants than by normally operating nuclear plants. Radionuclides – including isotopes of uranium, thorium, tritium, argon, noble gases, iodine, radon, and polonium – are released into the atmosphere from operating the various facilities. These radionuclides are dispersed into populated areas where exposure occurs by breathing or swallowing the materials. When coal is burned, the mineral content is converted into ash and slag. These wastes contain most of the radionuclides originally present, but a fraction of the ash is released into the atmosphere. The quantity released depends on the particulate control system, furnace design, mineral content of the coal, and existing emission control standards.

In the production of electrical power, coal is burned in furnaces operating at temperatures up to 1700°C. Most of the mineral matter in the coal is fused into a vitrified ash. A portion of the heavier ash, along with incompletely burned organic matter, drops to the bottom of the furnace as bottom ash or slag. The fly-ash, however, is carried through the boiler along with the hot flue gases and any volatilized mineral compounds to the stack where, depending on the efficiency of emission control devices, some fraction is collected while the rest of the escaping fly-ash is released to the atmosphere.

The amount of emission of radioactive isotopes into the atmosphere through the power-plant chimney depends mainly on the efficiency of electrostatic precipitators or other devices used for cleaning the flue gases. There are two types of coal-burning power plants in operation [6].

- Power plants with emission into the atmosphere of about 10% of the produced fly-ash;
- modern power plants with emission of only about 1% of the produced fly-ash.

The arithmetic mean for potassium-, uranium- and thorium-activity concentration in fly-ash carried by flue gases through electrostatic filters into chimneys is:

^{40}K   500 Bq kg^{-1}
^{238}U   200 Bq kg^{-1}
^{232}Th   200 Bq kg^{-1}

Based on these values one can obtain the estimate of atmospheric release. On average, it is:

^{238}U   1500 MBq GW^{-1} year^{-1}
^{232}Th   1500 MBq GW^{-1} year^{-1}

Phosphate rock deposits contain uranium (U), radium (Ra), thorium (Th), and other radio-nuclides as contaminants. Uranium in phosphate rock deposits throughout the world at a range from 3 to 400 mg kg^{-1} [7]. It has been estimated that 1000 kg of Florida phosphate rock contains about 100 μCi each of ^{238}U and ^{226}Ra and 4 μCi of ^{230}Th [8]. Some of these elements are retained in the H_3PO_4 and the remainder are transferred to the by-products during fertilizer manufacture. For example, it is estimated that 60% of the radioactivity in the mined Florida phosphate rock remains with slime and sand tailings during beneficiation [9].

Literature on the radionuclide content of phosphates is rather extensive. Radium, uranium, thorium and members of their decay series are the principal radioelements present in fertilizers. The radium content in fertilizers has been discussed extensively [10,11]. Other reports deal with radon release owing to the use of phosphate fertilizers [12,13]. However, the majority of authors are concerned with uranium and thorium concentrations.

Sedimentary phosphate ores, such as those found in Florida and Morocco, tend to have high concentrations of uranium, whereas the opposite is the case with magmatic ores, such as apatite from Kola. Typical activity concentrations of ^{238}U are 1500 Bq kg^{-1} in sedimentary phosphate deposits and 70 Bq kg^{-1} in apatite. ^{238}U is generally found in radioactive equilibrium with its decay products. The activity concentrations of ^{232}Th and of ^{40}K in sedimentary phosphate rock are much lower than those of ^{238}U, and comparable to those observed normally in soil. Several authors have reported on uranium concentrations in phosphates [14–18] Radioactivity in phosphate rocks owing to ^{238}U, ^{226}Ra, ^{232}Th and ^{40}K has been discussed by some authors [19,20] while others have discussed U and Th activity as a function of particle size [21,22]. Radioactivity released by the use of phosphate fertilizers, its accumulation in soil, and the migration and transfer of radioactivity into plants is extensively discussed [23–30].

The mining and processing of phosphate ores redistribute ^{238}U and its decay products among the various products, by-products and wastes of the phosphate industry. Effluent discharges into the environment, as well as the use of phosphate fertilizers in agriculture and of by-products, in the building industry are possible sources of exposure to the public [31,32].

The exposure pattern, or chain of relationships between radioactivity in agricultural products and the radiation dose and health effects (risk) in humans are presented schematically in Fig. 11.1. The actual radionuclides present in some phosphate fertili-
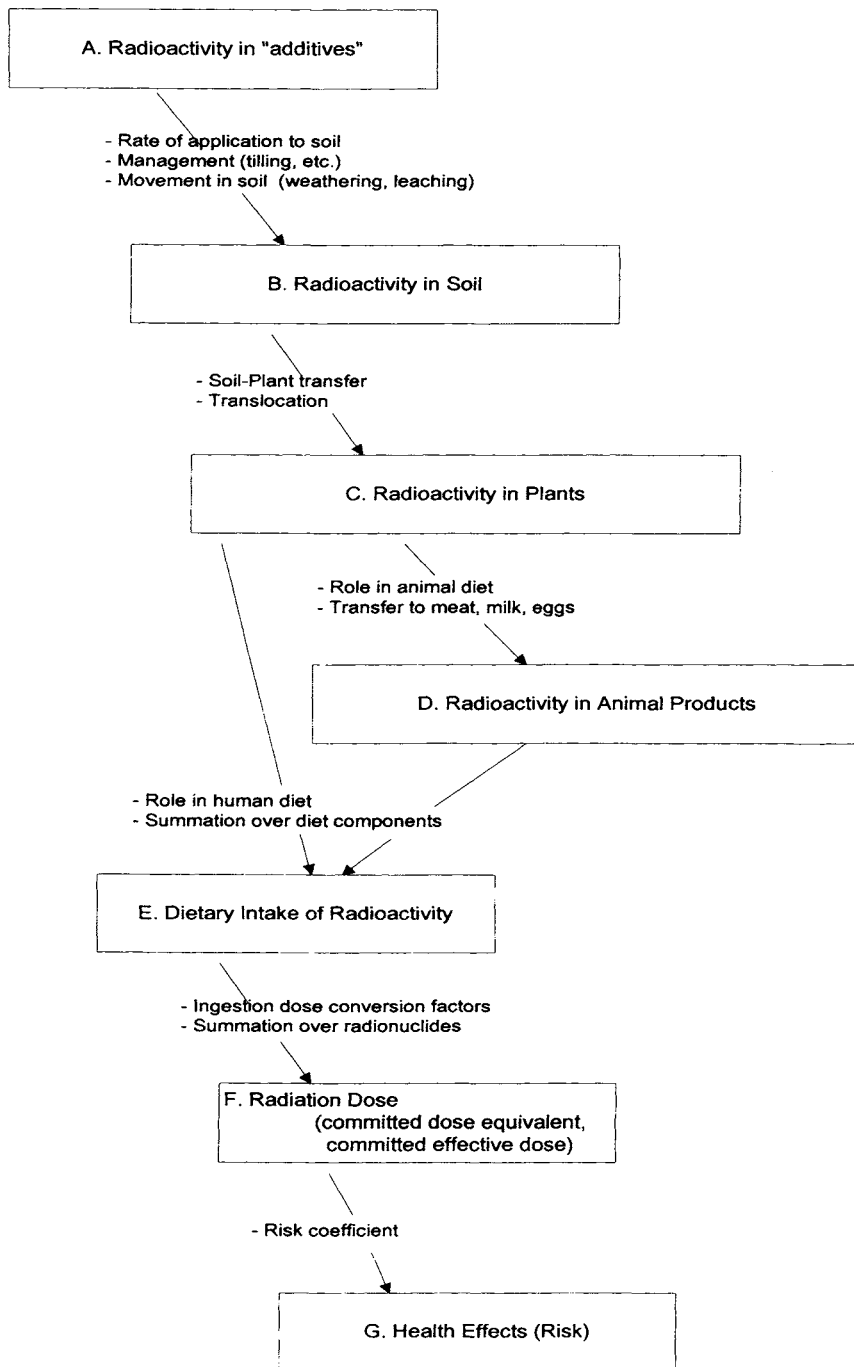
Fig. 11.1. Chain of relationships; radioactivity in agricultural 'additives' to dose and risk in humans.

zers (including by-products and wastes) contribute to the radiation exposure of a population [33–35].

Phosphate rocks vary considerably in their content of U, Ra, and Th, depending on the geographical area from which they were mined. In a survey of phosphate rock samples from all of the major phosphate rock-producing regions of the world at the time, the median contents were 59 mg kg^{-1} of U and 8 mg kg^{-1} of Ra [8]. It was estimated that totals of 400 μCi each of U and Ra, and 15 μCi of Th, had been applied per acre in some potato (*Solanum tuberosum* L.) fields in Maine over a 45-year period. Most crops are not as heavily fertilized potatoes as with P, although tobacco (*Nicotiana tabacum L*) is usually highly fertilized with P. About 33% of the U in beneficiated phosphate-rock concentrate-feed for acidulation by the wet-process method was found in the phosphogypsum (PG) by-product. The remainder of the U was found mainly in the H_2PO_4, which is subsequently processed into several types of P fertilizers.

Geothermal energy is produced in Iceland, Italy, Japan, New Zealand, the USSR and the United States. At the present time, it accounts for only 0.1% of the world's energy production [36] but its relative importance may grow in the future, as the potential resources of geothermal energy are believed to be very large. In geothermal energy extraction, use is made of hot steam or water derived from high-temperature rocks deep inside the earth. The geothermal fluids carry natural radionuclides, and especially ^{222}Rn, which is discharged into the atmosphere. From measurements of ^{222}Rn activity concentrations in the hot stream used in three Italian power plants, the ^{222}Rn annual releases have been estimated to be 110 TBq from the 400 MW Larderello plant, 7.0 TBq from the 15 MW plant at Piancastagnaio, and 1.5 TBq from the 3 MW plant at Bagnore [37]. These figures point to an average ^{222}Rn atmospheric discharge per unit energy generated of about 400 TBq per GW a. The corresponding collective effective dose-equivalent commitment per unit energy generated is estimated to be about 6 man Sv per GW a, if the assumptions used for the discharges from coal-fired power plants are applied. It may be recalled that these assumptions are: an equilibrium factor of 0.6 between ^{222}Rn and its short-lived decay products; population density of 100 km^{-2} around the plant; effective dose equivalent per unit activity inhaled of 1.3×10^{-8} Sv Bq^{-1}; indoor concentrations equal to the outdoor concentrations.

Annual individual effective dose-equivalents resulting from inhalation of short-lived decay products of ^{222}Rn have also been estimated. Assuming, as in the case of discharges from coal-fired power plants, an effective stack height of 100 m, and an annual average of the ground-level air concentration per unit release rate of 4×10^{-8} Bq m^{-3} per Bq s^{-1} at 1 km from the stack, the annual effective dose equivalent resulting from atmospheric ^{222}Rn discharges would be about 3×10^{-5} Sv for an individual of the critical group living around a geothermal plant of 1 GW of electrical power. It is to be noted that the existing geothermal plants have a lower power, resulting in correspondingly lower estimates of annual effective dose equivalents.

The consumer products containing deliberately incorporated radionuclides can be broadly classified into five categories; radio-luminous products [38]; electronic and electrical devices [39]; antistatic devices [40,41]; smoke detectors [42,43]; and ceramics, glassware [44], alloys, etc., containing uranium or thorium. Some of these products, such as the antistatic devices, are more widely disseminated in industry than among the general public.

11.4 PATHWAYS AND SAMPLES OF INTEREST

In this discussion, we first discuss food items. In this we follow IAEA Report No. 295 [45] and describe samples and pathways relevant to the analysis of radionuclides in foods, and of environmental materials that are part of the immediate pathways leading to contamination of food.

The source of a release, and the conditions at the site where it occurs, determine one or more critical pathways in the environment between the point of discharge and man. The season of the year determines to a great extent the extent of contamination of various foods or environmental components.

The main purpose of analysis should be fast identification of the most critical samples and the most important radionuclides, so that the necessary actions can be carried out rapidly. Only those foods should be sampled, and those radionuclides analysed, whose consumption contributes significantly to population exposure. If, for example, ^{131}I is being released in proximity to cow pastures, its concentration in the milk produced will provide far more meaningful information than its concentration in air, or deposition on forage samples. Nevertheless, measurements of ^{131}I in pasture grass may be very important in providing an indication of the expected concentration in milk. For other circumstances, the need for food sampling should be based on a thorough understanding of agricultural practice and of food consumption in specific areas of interest.

It is recommended that food analyses be based on the determination of radionuclides in individual food items rather than a mixed diet sample. Only the analysis of individual foodstuffs can indicate whether and which countermeasures should be taken to reduce doses. Food sampling for estimation of the total consumption should be carried out at the retail level when appropriate; otherwise it should be carried out at the consumption level. The selection of foods to be sampled can be based on individual diet or food-consumption statistics. The analysis of individual foodstuffs should preferably be performed after preparation, taking into account the effect of kitchen activities such as washing, cleaning and cooking.

Milk and milk products are important components of the diet in many countries. Milk is one of the few foods produced over large areas and collected on a daily basis. Its composition is almost identical all over the world, and it is easy to collect a representative sample that can be analysed in liquid or dried form. Milk is likely to be contaminated by radioactive iodine and caesium within the first days after a release of volatile radionuclides. Contamination of milk will be greatest when cows are grazing during the fallout period, but even when cows are kept indoors the contamination of milk may occur by inhalation of radionuclides or their ingestion via drinking-water or contaminated feed. Milk from goats and sheep should be checked periodically over a longer period, because of their grazing habits.

After harvesting, grain and rice are subjected to contamination only during storage, and only the outer layers would be contaminated. If fallout occurs during the growing season, the radionuclides will be transported into the grain and rice through the plant growth process. It is relatively easy to select representative samples of grain and rice at harvest time. If the fallout occurs during the winter, the grain will be contaminated only through root uptake in the next growing season.

Following an accidental release of radiocaesium, meat becomes one of the main sources

of dietary contamination. This mainly results during animal grazing, but contaminated drinking-water might also be an important pathway. Inhalation of radiocaesium is not likely to be a significant pathway for meat. Meat sampling should normally be done in such a way that the composite sample is representative of a large number of animals, although screening measurements of individual animals may be necessary after heavy fallout.

Following an accident, contamination of fish in nutrient-deficient lakes may constitute a particularly significant pathway to the uptake of radiocaesium by man. Obtaining a representative sample from an area containing many lakes may require some compromise, since the collection of samples from a large number of the lakes may be impracticable. Ocean fish will not take up as much radiocaesium as freshwater fish because of the dilution through the depth of the ocean and the effective dilution associated with the high potassium content in the water. Particulate-associated radionuclides can, however, be enriched to high levels. Mussels such as *Mytilus edulis*, some species of macroalgae, and other filter-feeders quickly take up the contaminants from sea-water and can also be used as biological indicators.

Green leafy vegetables are very prone to external contamination during their growing season. Other vegetables, including root vegetables, may also become contaminated. It is important to obtain representative samples, and the sampling should be planned carefully. In the early stages of fallout, green vegetables can provide very significant pathways for short-lived radionuclides.

Game, and foods such as mushrooms and berries, can be contaminated markedly, although they would contribute significantly to the ingestion dose only in very rare cases. It may still be advisable to analyse these foods in order to decide whether the levels comply with international export regulations.

Environmental samples to be analysed for the activity of the various radionuclides include air, water, soil, grass and sediment.

Measurement of airborne radioactivity provides the first opportunity of identifying the spectrum of radionuclides making up the contamination. Radionuclides will appear very rapidly in ground-level air, and air samples can give the first indication of the nature of the contamination. Radioactive materials in the air may result in exposure to man by inhalation, by ingestion of particulate matter deposited on vegetation, or by ingestion of products derived from animals which were exposed to radioactive materials through inhalation or ingestion.

Rain-water and snow are also early indicators of radioactive contamination. In some places, drinking-water and rain-water can be significant pathways of short-lived radionuclides such as radioiodine to man or animals. Drinking-water and household water represent potentially important pathways, directly or through their use in food preparation and processing, although dilution, time-delays and water treatment can reduce the contamination levels markedly. Water consumed by livestock and/or used for irrigation purposes can also be a source of radionuclides in foods. Sea-water can be a source of contamination for seafoods such as mussels, shellfish, fish and algae. Water from streams, lakes and ponds should also be considered as a source of contamination.

Contaminated soil serves as a direct source of radionuclides, leading to the contamination of all agricultural products. Contaminated soil used in greenhouses could add significantly to the contamination of vegetables.

Grass is a direct pathway of radionuclides to animals and then to man through meat and/ or milk. The radionuclide content of grass can provide a basis for deciding whether cattle can be permitted to graze in a given area.

Sediment in all types of water (sea, lake, pond and large or small streams) may be a source of contamination for aquatic organisms. Contaminated sedimentary materials used as fertilizers may also increase the radioactivity levels of soil.

Following a release of radionuclides from a uranium-fuelled reactor to the environment the most important radionuclides to be assessed for internal exposure from the ingestion of food and water, and for the contamination of environmental materials which are parts of the immediate pathways leading to contamination of food, are ^{134}Cs, ^{137}Cs (^{137m}Ba), ^{131}I and other gamma-emitters, the beta-emitters ^{89}Sr, ^{90}Sr and tritium, and the alpha-emitters ^{238}Pu, $^{239+240}$Pu, ^{241}Am and ^{242}Cm.

The levels of radionuclides in the environment and food have been extensively compiled by UNSCEAR [3]. In general, the radionuclides of major importance in the contamination of food and environmental samples (materials which are part of the pathways leading to the food) are:

Air	^{131}I, ^{134}Cs, ^{137}Cs
Water	^{3}H, ^{89}Sr, ^{90}Sr, ^{131}I, ^{134}Cs, ^{137}Cs
Milk	^{89}Sr, ^{90}Sr, ^{131}I, ^{134}Cs, ^{137}Cs
Meat	^{134}Cs, ^{137}Cs
Other foods	^{89}Sr, ^{90}Sr, ^{137}Cs
Vegetation	^{89}Sr, ^{90}Sr, ^{95}Zr, ^{95}Nb, ^{103}Ru, ^{106}Ru, ^{131}I, ^{134}Cs, ^{137}Cs, ^{141}Ce, ^{144}Ce
Soil	^{90}Sr, ^{134}Cs, ^{137}Cs, ^{238}Pu, $^{239+240}$Pu, ^{241}Am, ^{242}Cm

This group of radionuclides is most likely to be of concern in terrestrially produced foods. Biological concentration processes in freshwater and marine systems can result in very rapid transfer and enrichment of specific radionuclides. The radionuclides which enter such systems can in certain cases be accumulated rapidly by plankton and algae. These organisms serve as food for higher tropic levels and thus the radionuclides become concentrated in organisms such as oysters, clams and shrimps. Radionuclides of particular concern in freshwater and marine food chains include: ^{54}Mn, ^{55}Fe, ^{59}Fe, ^{60}Co, ^{65}Zn, ^{95}Zr, ^{95}Nb, ^{103}Ru, ^{106}Ru, ^{110m}Ag, ^{125}Sb, ^{131}I, ^{134}Cs, ^{137}Cs, ^{141}Ce, ^{144}Ce, and some of the transuranic elements.

Many other radionuclides would be present in debris from a nuclear accident, and their potential contribution to human exposure depends on the type of accident and the circumstances when it occurred. Since there are several types of fuel, the spectra of radionuclides that would be present in accidental releases could be somewhat different. The following four nuclear accident scenarios are considered in details in the IAEA Report No. 295 [45]:

- reactor meltdown, with or without failed containment;
- reactor meltdown with particle containment;
- nuclear fuel-reprocessing plant release;
- plutonium fuel-fabrication plant release.

The types and quantities of radionuclides released in each of these scenarios are different. Other nuclear accidents which may result in major atmospheric emissions are:

- plutonium fuelled reactor meltdown;

- breeder reactor meltdown;
- high flux radionuclide production reactor meltdown;
- fast flux reactor meltdown;
- nuclear-powered ship/submarine reactor meltdown;
- satellite re-entry and burnup of satellite nuclear power source;
- nuclear weapon destruction by chemical explosion;
- criticality at nuclear materials processing plant;
- fusion reactor fuel loss.

Each of these possible accidents may release a unique spectrum of radionuclides, and this should be considered in developing radioanalytical capabilities.

11.5 COLLECTION AND PREPARATION OF SAMPLES

Collection of samples, or sampling, is the method (or procedure) for extracting samples for the purpose of measuring the characteristics which are surveyed. Environmental radiation monitoring is mainly conducted with the aims of estimating an exposure dose for people near nuclear power facilities and of protecting public health and safety. In this case it is necessary to determine monitoring items, emphasizing the processes that result in individual exposure. These are based on the behaviour of radionuclides and on information about the population distribution, topography and geology, atmospheric phenomena, and, e.g., the types, quantities and intake of foods. There are also some general procedures that one should follow. For example, double identities should be placed on samples at collection time. It is advisable to fill in a standard form with all relevant information (date, location, fresh weight, weather, collector's name, etc.).

After collection, the samples must be stored properly to avoid degradation, spoiling, or other decomposition, and to avoid contamination. Proper care must be taken to avoid loss of volatile radionuclides. Short periods of storage before analysis may require refrigeration, or the addition of a preservative such as sodium bisulfite, alcohol, or formalin (as in the case of milk) for biological samples. When long periods of storage are needed, it may be preferable to convert the samples into a more stable form immediately after sampling. Drying or ashing of the sample will permit extended storage, but the temperature must be controlled carefully in these operations to avoid loss of radionuclides.

Sample-containers must be suitable for storage without degradation, especially when acids are added to liquid samples. The absorption of radionuclides from solution is less on polyethylene than on glass. With a few exceptions, almost all sorption losses can be eliminated by the addition of acid, a carrier solution containing stable elements, or a complexing agent. The sample-collection equipment, containers, and sample-preparation areas must be kept clean to avoid contamination. Disposable containers should be used whenever possible (plastic bags, aluminium trays, etc.).

Drying reduces the weight and volume of the samples and may also permit a longer storage time. Samples may be dried in a low-temperature oven at 105°C or at room temperature, without significant loss of any radionuclides except iodine. Samples should be dried for a sufficient period of time, at a fixed temperature, to acquire a constant dry weight. Measurements of fresh- or wet-weight and the dry weight are required. It is important to prevent contamination during the drying procedure. If necessary, freeze-

drying may be used to reduce further the loss of volatile radionuclides from the sample. However, this process is very time-consuming and is therefore not recommended highly.

Evaporation is the normal method of concentrating liquid samples. Reasonable care is required when evaporating liquids, particularly milk, with a hotplate in order to avoid spattering and loss of sample. Evaporation lamps usually eliminate the problem of spattering. The evaporation bowl should be made of material that will not absorb the radionuclides. Some radionuclides, such as iodine, tritium and ruthenium, may be lost during the evaporation process. A fast evaporation can be performed satisfactorily using a rotating evaporation system that operates under reduced pressure. Various volumes of the rotating spheres of up to 30 litres are available.

Where samples need ashing, low-carbon-nickel trays are adequate for the ashing operations. However, other trays lined with thin-sheet aluminum, which is discarded after each use, may be entirely satisfactory. Trays are easily cleaned with detergents or dilute mineral acids (usually HCl). The temperature for dry-ashing varies, but an upper limit of 450°C is recommended. If the sample is not completely dry at the start, an initial drying step at 105°C should be introduced. The ashing time depends on the type and quantity of the material; large samples may require 16–24 h. Dry-ashing should be used only for radionuclides that do not vaporize at the ashing temperature. Significant loss of caesium will occur above 400°C.

Carrier elements and radioisotope tracers should be added to all sample types before ashing. Measurements of the ashed weight are necessary for calculation of the radionuclide concentrations and yields. We now consider some specific materials in more detail.

11.5.1 Air

Air provides an important pathway through which humans are exposed, by inhalation, to a number of radionuclides. Air also conveys airborne radionuclides that were once sedimented in soil or on plants. Radionuclides that then reach humans through the respiratory system, digestive system, or skin cause both internal and external exposures.

For the analysis of radionuclides in airborne dust, the dust is collected on a filter using a dust sampler. Iodine in the air is collected on an active carbon filter using a dust sampler. Tritium exists in the form of vapour (HTO) or gas (HT) in the air. The HTO is absorbed on silica gel: HT is changed to HTO using a palladium catalyst and then the HTO absorbed on silica gel. Radioactive noble gases such as ^{85}Kr are absorbed on an active-carbon trap cooled with liquid nitrogen.

Several types of filter material are used for collecting aerosol materials (glass, PVC or Microsorban filters). All commercial filter media, when used properly, have adequate efficiencies. The filters are usually compressed to provide a standard counting geometry, and are measured by gamma spectrometry, after which they may be dry- or wet-ashed for radiochemical analysis.

Air particulate samplers are usually classified as low-volume air samplers or high-volume air samplers. There are, in addition to these classifications, dust samplers that consist of a combination of a low-volume suction pump and a movable filter-paper system. Characteristics of these samplers are as follows:

- *Low-volume air sampler.* A low-volume air sampler is an apparatus having a suction

capacity of up to 20 l/min. It is used for one continuous sampling lasting from several days to one week. Filter papers having a diameter of 5 cm and an active-carbon cartridge can be attached as a collecting device.

- *High-volume air sampler.* A high-volume air sampler is an apparatus whose suction capacity is between 500 l/min and 2000 l/min. It is used for a sample period of 1 day. A filter paper of dimensions 203 × 253 mm^2 (8 × 10 inches^2) can be attached as a collecting device.
- *Dust sampler.* A dust sampler has a suction pump with the same volume as a low-volume air sampler. It is capable of continuous sampling during 1 month when using an attached long filter paper. Most dust samplers used by local self-governing bodies are specially made to have an attached active-carbon cartridge in addition to the long filter paper. They are capable of measuring total beta and alpha radioactivities and the iodine content of the air.

In addition to a proper choice of collecting material (filter paper), a reliable measurement of flow rate is required. Flowmeters are classified into rotameters and integrating flowmeters. The latter are further classified into wet-gas meters and dry-gas meters. A rotameter has a specially graduated vertical tube, whose diameter increases in the ascending direction, containing a spinning top-shaped or spherical float. A gas stream is admitted into the bottom of the tube and the float is held at a vertical height which varies in proportion to the flow rate of gas.

11.5.2 Water

Tap-water should be collected at the water processing (filtration/purification) plants just prior to discharge into the distribution system. If the water is to be collected from a residence, then the pipes should be flushed sufficiently (2 or 3 min) prior to sample collection.

Rain collectors 0.1–1 m^2 in area provide for adequate collection of rain-water. Automatic sampling devices are available commercially which protect the collector from dry-deposition prior to the rainfall. These samplers start to open the collection area when rain begins to fall and close it when the rain stops. High-walled vessels with smooth surfaces are equally suitable. Some loss of the less-soluble radionuclides will occur on either of these collectors, but the loss can largely be recovered (if desired) by washing with dilute acid (0.1 N HCl). An alternative method is to filter the water directly through a mixed-bed ion-exchange column, after which the water is drained away. Contamination of rain-water samples by airborne soil and surface dust can be minimized by locating the sampling stations on the roofs of buildings. Overhanging vegetation should be avoided. The most suitable size for the collector depends upon the amount and frequency of precipitation in the area, as well as the frequency of collection.

If water samples have to be stored for any length of time, hydrochloric acid (11 M) should be added to the sample bottles at the rate of 10 ml per litre of sample, either prior to sampling or as soon as possible afterwards to avoid absorption of the radionuclides on the walls of the container. The longer is the storage time before analysis, the more important it is to acidify the water samples.

In addition to the radioactivity analysis of the samples, other information is required, including the:

- atmospheric conditions (water and surface-air temperature);
- water temperature, pH, salinity and degree of clarity;
- location (direction and distance from a navigational mark), latitude.

11.5.3 Soil

It is important to identify the radioactive concentrations in soil because it constitutes a path for radioactivity to humans, animals and plants, and is an indicator of radioactive accumulation in the environment. Soil includes submarine sediment and river-bed soil, in a broad sense, but here it includes only soil from uncultivated and cultivated land. The soil to be measured should consist of particles having diameters of 2 mm or less.

Sampling locations should not have obstacles nearby (trees, structures) and the utilization of the land should be considered. Also, sampling locations should not have unusual soil quality or topography, and should have little vegetation. Locations should be selected for periodic sampling to be possible, in order to determine the accumulation of radioactivity. Samples of earth transported from another place should be avoided, even when the soil has been mixed. Maps of the sampling locations should be sketched, or photographed whenever possible. Samples should be collected from the surface layer 0–5 cm deep using, at five to eight locations, a soil-sampler having a diameter of 10 cm.

Submarine sediment is an important material for helping the understanding of the accumulation of radionuclides discharged with wastewater from nuclear power facilities. Grains analysed should have a diameter of 2 mm or less.

Samples should be collected at the outlet of a facility drainage duct. Also, supplementary survey samples should be collected offshore. One should refer to marine charts or consult fishermen who are familiar with the region, because sampling may sometimes be hindered by a bedrock, even though the sampling location may have been selected with consideration of the ocean currents.

11.5.4 Biota

Plants take in radionuclides which have been discharged into the environment. In turn, people eat these plants or take them in through animals that have, directly or indirectly, eaten the plants. It is therefore important to measure the concentration of radioactivity in plants and animals when evaluating the exposure-doses of humans. Measurements should also be made on indicator plants and animals, which are neither edible nor directly involved in the human food chain. These indicators grow readily, concentrating radionuclides and when near nuclear power facilities-are very useful for monitoring changes in the level of environmental radioactivity.

Next we describe some of the objectives used in sample pretreatment. The objective of these procedures is to reduce the volume of the samples. Portioning, evaporation concentration, chemical separation, absorption, and so forth, are techniques which can be used alone or in appropriate combinations for liquid samples. Drying, sieving, pulverization, mixing, reduction, ashing, etc., are used alone, or in an appropriate combination, for preparing samples of solids for measurement.

The sample pretreatment procedures used will depend on the type of samples and of the activity to be measured. We shall mention some of the pretreatment procedures for γ-ray

Soil sample

↓

Weighing (wet weight)

↓

Drying (105°C)

↓

Weighing dried soil (dried soil weight)

↓

Sieving (2 mm and under)

↓

Weighing (dried fine soil weight)

↓   _____

                                         ↓

Pulverization (top Grinder)

↓

Weighing   no treatment for sand

↓   _____

                                         ↓

Mixing (V blender, etc.)

↓

Sample for analysis and measurement
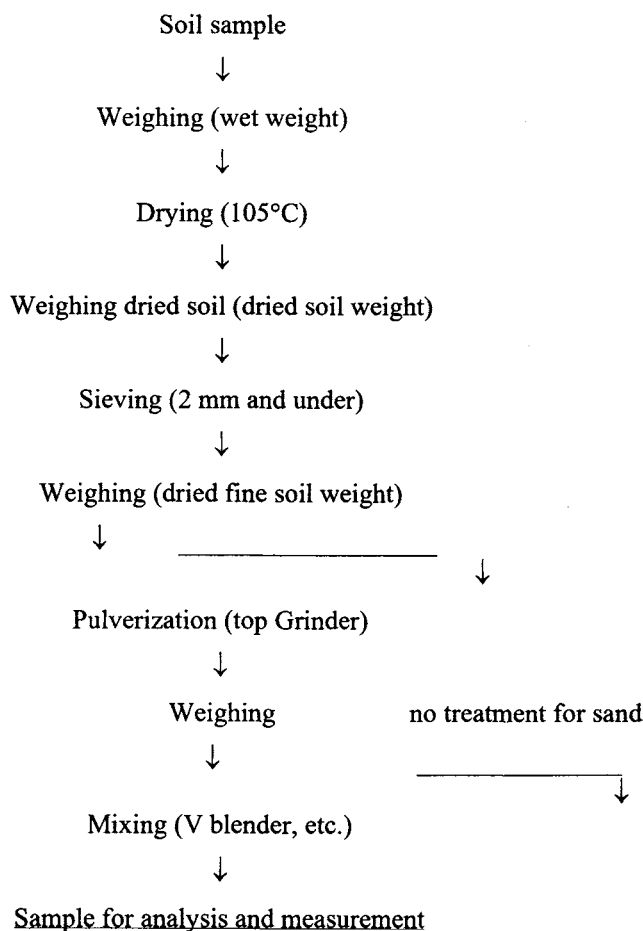
Fig. 11.2. Pretreatment of soil samples.

spectroscopy. As examples, Figs. 11.2 and 11.3 show the sequence of steps to be taken during preparation of soil samples and agricultural product samples for gamma spectrometry.

11.6 MEASUREMENTS OF RADIOACTIVITY

11.6.1 Counting

The fundamental statistical treatment by Currie [46] shows that the (low) limit of detection is proportional to the square root of the number of background continuum counts under the peak region of interest, where the proportionality factor varies with the confidence level chosen. Since the detection limit is expressed in counts, a more interesting parameter is the Minimum Detectable Activity (MDA), expressed in becquerels, and defined as the smallest quantity of radioactive nuclide which can be determined reliably,

Agricultural products

↓

Weighing

↓

Pretreatment (washing with water, removing roots or cores, peeling, etc.)

↓

Fresh weight measurement

↓

Portioning (put 1 Kg of each sample in a 30 mm round-bottomed porcelain vessel).

↓

Drying (in a hot air drier at 105°C)

↓

Weighing ash (drying rate)

↓

Carbonizing and ashing (450°C, 24 hours)

↓

Ash weight measurement (ash content percent)

↓

Sieving and mixing (screening at 0.35 mm)

↓

Sample for analysis and measurement

Fig. 11.3. Pretreatment of samples of agricultural products.

given the prevailing conditions of the specific spectral measurement. The MDA is inversely proportional to the absolute detection efficiency at the full-energy photo-peak. Smaller MDAs can be obtained by lowering the background and increasing the detection efficiency. (see also the books by Watt and Ramsden [48]; Friendlander et al. [47]).

For the measurement of the concentrations of radionuclides in environmental samples, and especially for low concentration levels, it is essential to reduce the background rate, as well as improving the counting efficiency.

In our consideration of the background-rate reduction, it is important to pay attention to the origins of the background. These are:

• natural and artificial radioactivity in the environmental circumstances;
• radioactivity in the detector and/or shielding material;
• cosmic radiation;

- instrumental noise.

In the environmental materials – for example, a concrete wall of a building – surrounding the soil, air or water, there are various kinds of natural and artificial radionuclides which give rise to a background. The most important are ^{40}K, ^{226}Ra and its decay products, and ^{232}Th and its decay products. As a result, many lines originating from these nuclides are observed even in a heavy shielding box, the spectrometry with a Ge detector. The background owing to these radiations can be reduced considerably by shielding with heavy materials such as lead and iron. Typically, such background can be reduced to a hundredth by a 10 cm thickness of lead or 30 cm of iron. In making a shielding box, it is important that the detector is surrounded entirely (4π direction) with shielding. In choosing the shielding material, one has to pay attention to any undesirable contamination with radioactive substances. For example, a small amount of ^{210}Pb(RaD), which is a member of the ^{226}Ra series, is inevitably contained in lead. Its daughter nuclide ^{210}Bi(RaE) emits energetic β-rays ($E_{max} = 1.17$ MeV), resulting in Bremsstrahlung which may contribute to an increase in background rate. The concentration of ^{210}Pb is very dependent on the mine where it was produced, and a careful check on the content of ^{210}Pb is important for achieving ideal shielding characteristics. Since the half-life of ^{210}Pb is 22 years, old lead has much better characteristics. Iron blocks are sometimes used instead of lead. However, the modern iron/steel is often contaminated with ^{60}Co, and careful checking is necessary prior to the construction of the shielding assembly. This ^{60}Co contamination originates from the ^{60}Co source which was used to monitor the abrasion of the furnace wall. For shielding purposes, the use of old iron, for example from materials from a sunken ship, is recommended. The background may also originate from the trace amount of radioactivity in the detector itself and its assemblies, and cannot be eliminated by shielding alone – hence, the contamination of the detector materials with radioactive substances should be examined carefully. A typical example is ^{40}K in the glass used for phototubes. This can be eliminated by the use of a quartz phototube. The ^{226}Ra present in solder sometimes gives rise to a considerable background rate. The molecular sieve used for keeping a vacuum in the cryostat of a Ge detector sometimes causes a background spectrum. In this sense, electrolytic copper, stainless steel, and Perspex are recommended as materials to be used in the shielding box. Even when very thick materials are used for shielding, it is very difficult to stop the penetration of highly energetic cosmic-rays, which have energies in excess of 10^3 MeV.

In order to overcome the undesirable background, an electrical guard technique is employed. The cosmic rays which give a pulse to the main detector should produce another signal to one of the guard counters. The two signals from the main counter and from the guard counter are produced simultaneously. Therefore an anti-coincidence technique is applied quite effectively to reduce the background rate, and an ultimate low background rate of the order of 0.1 cpm can be achieved by the combination of heavy shielding and the anti-coincidence guard technique.

The coincidence technique is often used for the low-level detection of electrons. As an example, we mention the low background β-ray spectrometer as developed by Tanaka [49]. A small Geiger–Müller counter is mounted inside a large plastic scintillator, and scintillation pulses from only those events which are coincident in the two detectors are analysed by a multichannel analyser. In this case, signals caused by cosmic rays are also

subject to the analysis, but the minimum width of the scintillator is designed so as to produce a very large pulse-height, and this signal is rejected in the course of the pulse-height analyses. In these instruments, the β-ray spectra can be measured for even very weak samples; for example, the β-rays originating from the ^{40}K contained in the ash of a 3-cm long cigarette. By the addition of a logarithmic amplifier, the analyses of β-ray spectra become even easier.

Pulse-shape discrimination is also used sometimes for background reduction. Electrical noise is very often caused by the discharge of high-potential circuits, the transition signal of thyristors, and the induction signal from spark-gaps, etc., which result in a background signal to the counter. The prevention of this noise, electrical filtering in the main power supply, and electrical and/or magnetic shielding are sometimes required.

Another essential requirement for reliable low-level counting is to increase the counting efficiency and/or to use a larger amount of source sample. In order to increase the counting efficiency, one should use a large-volume (large size) detector, and try to improve the source-to-detector geometry. However, it should be noted that with increasing detector-size or volume, the background may also increase. For the improvement of the geometry, the use of a well-type detector is advantageous, but the source size is sometimes limited. On the other hand, to use a larger amount of source, it is convenient to use a Malineli-beaker, especially for the measurement of environmental samples such as milk or soil.

In principle, one can perform the measurement of activity of any sample by using radiation detection systems and standard sources. Standard sources and solutions are available from many national and international operations. However, for sources one can perform radioactivity measurement without the help of standards.

Absolute counting implies a method of radioactivity measurement performed without the help of a standard/reference source. The methods for the absolute measurements can be classified into two categories. The first comprises the direct methods, which include the defined solid-angle method, the 4π-counting method, the internal gas counting method, and the liquid scintillation counting method. These methods use relatively simple procedures, and each provides unique applicability. However, some uncertain corrections, such as for the self-absorption, are involved in these methods, and the accuracies obtainable are somewhat limited. The second category is based on the coincidence technique, by which we can determine the radioactivity more accurately without any uncertain estimation of correction terms.

The methods most often used include: the 2π α-counting method, 4π β-counting with a 4π gas flow counter, 4π β-counting with a liquid scintillation spectrometer, and the 4π β-γ coincidence counting method.

11.6.2 Gamma spectrometry

High-purity detection systems having a very low background are suitable for the direct measurement of low-level radioactivity in environmental samples. The background features of the detection system are of considerable importance because they have to be known for one to obtain an estimate of the detection limit and of the minimum detectable activity [46]. The natural radioactivity background originates from the uranium and the thorium series from ^{40}K and from cosmic rays. Natural radioactivity is found in most materials, and it is necessary to shield the detector using carefully selected materials of

high density. The materials used in the detector assembly, and the shielding materials, need to have the lowest possible inherent background radiation [50]. Containers such as Marinelli-beakers can be filled with aliquots of environmental samples and placed on top of the end cap of the detector in an accurately reproducible beaker-detector geometry [51,52]. The radioactivity of the samples can be measured if the detector has been calibrated with Marinelli-beaker standard sources (MBSS) of the same dimensions, density and chemical composition. The calibration procedure should follow as closely as possible that defined in the IEC Standard 697 [53]. This method enables the simultaneous detection of several gamma-emitters present in the sample matrix without the need for separation of the radionuclides from the matrix.

The method can be applied to a large variety of environmental and biological materials such as air, water, soil, sediments, vegetation (grass, hay, etc.) and, particularly, individual foods of vegetable and animal origin as well as total diet mixtures. This method is suitable for the surveillance and monitoring of radioactivity originating from the operation of nuclear plants, nuclear weapons tests, and releases from nuclear accidents [54].

It is recommended that the gamma spectrometry system should be a fully integrated data acquisition and computation system comprising the following items [45]:

- A vertical, high-purity germanium (HPGe) detector is recommended. The detector should have an efficiency of 18–20%. Generally, the efficiency of germanium detectors is specified as the photo-peak efficiency relative to that of a standard 7.62 × 7.62 cm cylindrical NaI(Tl) scintillation crystal, and is normally based on the measurement of the 1.33 MeV γ-ray photo-peak of a ^{60}Co source with a source-detector spacing of 25 cm used in both measurement systems. The resolution of the detector, which is normally specified for germanium detectors as the full width (in keV) at half maximum (FWHM) of the full energy peak of the 1.33 MeV peak of ^{60}Co, should be between 1.8 keV and 2.2 keV. It is recommended that the peak-to-Compton ratio of the detector be greater than 46:1. The peak-to-Compton ratio is defined as the ratio of the count in the highest photo-peak channel to the count in a typical channel just below the associated Compton edge, and is conventionally quoted for the 1.33 MeV γ-ray photo-peak of ^{60}Co.
- A preamplifier is necessary. This normally an integral part of the detector unit and is located very near the detector in order to take advantage of the cooling which is necessary for the operation of the detector and which helps the preamplifier to operate with low noise.
- A biased high-voltage power supply is required to supply high voltage to the detector through the preamplifier. A power supply of 1500–5000 V is adequate for the operation of germanium detectors.
- A linear amplifier is required to process the output signals from the preamplifier.
- A detector shield will be needed with a cavity which is able to accommodate large (up to 4 l) samples, constructed of either lead or steel with some type of graded liner to degrade X-rays. Lead shields have a much lower back-scatter effect than steel shields. Typically, lead shields have walls 5–10 cm thick, lined inside with graded absorbers made of cadmium (ca. 1–6 mm) and copper (ca. 0–4 mm). Other materials, such as plexiglas and aluminium, are also used as absorbers.
- A multichannel analyser (MCA) with a minimum of 4096 channels should be

connected to a keyboard and display screen for input and output of data and interaction with a computer. Several kits are available for the conversion of personal computers (PCs) into MCAs. Basically, there are three types of conversion kits. One makes use of a board with an analogue-to-digital converter (ADC) that simply clips into the PC; a second type uses a clip-in board with an external ADC; and the third type uses a multichannel buffer (MCB) connected to the PC. All of these PC-based MCA systems are relatively inexpensive and are very suitable for use in germanium -and sodium iodide γ-ray spectrometry.

- A rapid data-storage and recovery system is needed. It can consist of magnetic tape, hard disk, floppy disk, or a combination of these media. This system can be used for programming, short-term storage of data, and archiving data.
- A high-speed printer is required for data output. Useful, but not absolutely necessary, is a plotter for archiving spectral drawings.
- Software for system operation and data reduction is usually supplied with the MCA system. Software packages with varying features and capabilities are available for MCAs based on PCs.

Several aspects of gamma spectrometry with such a system deserve some discussion. Interferences associated with gamma determinations may be caused by improper spectral identities, changes in background, errors in calibration and/or geometry, and lack of homogeneity in samples. Many of the problems in γ-ray spectrometry are due to malfunctions of electronic components. Very important also, is the calibration of the measuring systems; both energy- and efficiency-calibration should be performed with care.

Energy calibration of a germanium detector system (i.e., establishing the channel-number of the MCA in relation to a γ-ray energy) is achieved by measuring mixed standard sources of known radionuclides having well-defined energies within the energy range of interest, usually 60–2000 keV [55]. The use of the lower energy photons emitted by ^{241}Am may indicate changes in the intercept. Mixed γ-ray standards are available in various forms and containers from reliable suppliers. A partial list of radionuclides usually available with gamma energies in the range of interest includes: ^{241}Am, ^{109}Cd and ^{57}Co, ^{139}Ce, ^{51}Cr, ^{22}Na, ^{54}Mn, and ^{60}Co. The energy calibration source should contain a selection of radionuclides with at least four different γ-ray energies. It is recommended that one of the nuclides should be ^{137}Cs. The gain of the system should be adjusted so as to position the 662 keV photo-peak of ^{137}Cs at about one-third full scale. It is also recommended that the gain of the system be adjusted to 0.5 keV/channel. Once these adjustments are made, the gain of the system should remain fixed.

Most laboratories involved in radiation measurements now use personal computers and commercially available software for the analysis of γ-ray spectra. Some of the programs allow the user to control the multichannel analyser (MCA), calibrate the detector for various geometries, and provide analysis results. The programs are easy to use and do not require the user to be an expert in γ-ray spectrometry.

As an example we refer to work by Heimlich et al. [56]. In their work, the gamma-ray spectrum analysis program, GAMANAL, has been modified to operate on a microcomputer. The program uses an algorithm involving a Gaussian- and a tailing-term for fitting and resolving peaks obtained from spectrometers using germanium semiconductor detectors. Gamma-ray energies, intensities and absolute photon emission rates can be deter-

mined. A graphical output showing the original and fitted data can also be obtained. The results generated by the program are stored on disk as ASCII files for further analysis. This permits the use of other computer programs and languages in tasks such as decay-curve analysis, radionuclide activity measurements, and neutron activation analysis.

Sanderson [57] evaluated commercially available IBM PC-compatible software in 1987. At that time, it was reported that most of the programs detected peaks satisfactorily and resolved doublets of equal intensity. Problems arose when the doublets were of unequal intensity or the analysis of a complex spectrum was needed. The suppliers of the programs involved in that study have corrected some of these deficiencies. Since many of these programs have undergone numerous revisions, and a few new programs have become available, a re-evaluation has been performed [58].

Six programs were evaluated in the newer study [58]: GAMMA-W (Gesellschaft für Kernspektrometrie, Germany), INTERGAMMA (Intertechnique Instrumentation Nucleaire, France), QSA/Plus (Aptec Nuclear, Inc., Canada), OMNIGAM (EG&G Ortec, USA), GDR (Quantum Technology, Inc., USA), SAMPO 90 (Canberra Nuclear Products Group).

Hardware requirements were similar for all the programs tested (IBM PC compatibility, 384–640 K of memory, a hard-disk drive). Two programs, QSA/Plus and SAMPO 90, also required a maths co-processor. The QSA/Plus program had to be installed in Windows 3.0. All of the other programs operated directly from DOS. Except for GAMMA-W, all the programs control data acquisition. GAMMA-W, which is written by an independent company that does not manufacture nuclear instrumentation, does not allow the user to read ten different formats of γ-ray spectra. The conclusions reached by the authors were as follows:

All of the programs satisfactorily found small peaks using sensitivity values recommended in the program manuals. However, these values may not be optimal for every situation. When the sensitivity values were lowered, additional valid peaks were found. When the recommended sensitivity values were used, only two programs did not report any false peaks. All of the programs were able to resolve equal-intensity doublet peaks with only a 2 keV (4-channel) separation. The resolution of doublets of unequal intensity, especially where the smaller is on the high-energy side of the predominant one, has improved since the last evaluation. However, some programs still require improvement in this area.

All the programs for analysis of gamma spectra measured with germanium detectors generally allow the following steps (among others) to be carried out:

- transfer of spectra from the multichannel analyser to the computer;
- search for, and identification of photo-peaks;
- energy and efficiency calibrations;
- resolution of multiplets;
- calculation of activities and the dosimetric magnitudes deriving from them.

Most of these programs, however, are not specifically designed for the development of plans for environmental radiological monitoring, where one must measure periodically and systematically the activity levels of a set of man-made gamma-emitters in different types of environmental samples. Such samples often present very low counting statistics for these emitters.

Moreover, since most of these programs employ elaborate algorithms for the search-

and-fit to the shape of the gamma-peaks, they are coded in FORTRAN, and are therefore implemented on minicomputers or, in more reduced versions, on IBM-type personal computers. Also, commercially available programs are difficult for the user to modify, usually requiring a great deal of programming effort to adapt them to the peculiarities of each laboratory.

Baeza et al. [59] have developed ESPEC, a set of programs especially designed to undertake gamma spectrometric analyses of environmental samples. The processes listed above are carried out in a simple form. The package is designed so that it can be implemented not only on an IBM-type personal computer, but even on one with less memory and lower performance, such as a 64-kbyte memory microcomputer. The code is written in BASIC and is easily modified to suit the specifications of any multichannel analysers.

11.6.3 Beta-particle spectrometry

Table 11.5 lists beta-emitting radionuclides and since beta-emitters show a continuous emission spectrum, the average energy E_B, and the maximum energy, E_B^m, are given. Measurement uncertainties are shown in brackets after the respective value (in units of the last significant digits).

The classical way to measure low-level beta-particle activity is with a Geiger–Müller (GM) gas-flow counter. In anti-coincidence with a guard detector the GM gives a fairly low background (0.003 s^{-1}) and a counting efficiency of 40%. The disadvantage, however, is its inability to give energy resolution. As the characteristics of the GM tube may gradually change over a long period of use, it is necessary to examine the characteristics of the tube and background-count occasionally. The counting efficiency of a GM counter for detecting β-rays is defined as the ratio of the count rate to β-activity of the source. Thus, the counting efficiency is one of the most important factors to determine the characteristics of the detector.

There is the following relationship between activity A (Bq) and net count-rate, n (s^{-1}) in activity measurements using the GM counter

TABLE 11.5

SOME COMMON BETA-EMITTERS

Nuclide	Half-life	E_R^m	E_R (%)	E_r (keV)
[3]H	12.35 (1) a	18.6	5.68	
[14]C	5730 (40) a	156.48	49.47	
[32]P	14.29 (2) d	1710.4	695.0	
[35]S	87.44 (7) d	167.47	48.80	
[55]Fe	2.75 (2) a			K_α 5.9, K_β 6.5
[63]Ni	96 (4) a	65.87	17.13	
[89]Sr	50.5 (1) d	1492	583.1	
[90]Sr	28.7 (3) a	546.0	195.8	
[90]Y	64.1 (1) h	2284	934.8	
[125]I	59.3 (2) d			K_α 27.4, K_β 31.0
[210]Pb	22.3 (2) a	16.5	4.15 (80)	
		63.0	16.13 (20)	

$$n = A f_s f_i f_{sa} f_s f_a \tag{1}$$

where f_s is geometry factor of counting setup; f_i, intrinsic efficiency of GM tube for β-rays; f_{sa}, source self-absorption factor; f_s, source-mount backscattering factor; f_a, correction for absorption between the source and GM tube.

The above equation may be rewritten by replacing f_s, f_i, f_{sa}, f_s with η, where

$$\eta = \frac{n}{A f_a} \tag{2}$$

is the counting efficiency; it represents the count-rate, n, corrected for the adsorption f_a to activity A.

The counting efficiency, η, is obtained by using standard source of the known activity A, and the absorbers with the known thickness d_m. If the count rates for β-rays are defined as n and n_0, respectively, which correspond to those passing through with-and without absorber, f_a is equal to n/n_0, where f_a represents the transmission rate of β-rays for the absorber, thickness d_m, When the thickness of absorber d_m is not so large compared with the maximum range of β-rays, n can be represented by the experimental formula

$$n = n_0 e^{-\mu_m d_m} \tag{3}$$

Therefore, if one plots the n values as a function of the thickness d_m on semi-log graph paper, the n values decrease linearly as the d_m values increase. The slope of this line is μ_m, which is called the mass absorption coefficient. If one puts $d_m = 0$, the count rate n_0 ($= n/f_a$) can be obtained. Thus the equation becomes

$$\eta = \frac{n_0}{A} \tag{4}$$

If η is already known, the unknown activity A' is obtained by

$$A' = \frac{n'_0}{\eta} \tag{5}$$

where n'_0 is the count rate extrapolated to $d_m = 0$.

Furthermore, in a case where the actual count rate n is larger than 100 s^{-1}, it is necessary for the count rate to correct the resolving time τ (s) of the GM counter.

It has been shown [60] that ion-implanted detectors can be used not only for alpha-particle spectrometry but also for beta-particle spectrometry. Their drawback, however, is the high background around 100 keV and noise below 1 keV.

Olssen et al. [61] have described a detector system for low-level beta-particle spectrometry where the good characteristics of gas flow and silicon detectors are used. The gas-flow GM counter used in the detector system is a windowless single-channel version of the GM-25-5 multicounter developed in the Riso National Laboratory, Denmark. The gas-flow counter utilizes a GM counter with a diameter of 22 mm and a guard of $80 \times 90 \times 10$ mm, using argon (99%)/isobutane (1%) as counting gas. Background counts, produced in the sample counter by cosmic radiation, are reduced by means of the guard and attached anticoincidence circuits. As the energy-discriminating detector a passivated implanted silicon detector, with an active area of 450 mm^2 and a depth of 300 μm, was used. The PIPS detector has a 0.5-μm thick aluminium coating on the front surface. The detector is

placed on top of the gas-flow counter, and integrated into the gas detector unit, allowing the aluminized front surface to act as one of the ground-electrodes of the sample counter [60]. The source is placed in a cavity inside the detector, between the sample and guard counter. The instrumentation used for PIPS detectors is identical to that used for alpha-particle spectrometry.

Holm et al. [60] have demonstrated that it is possible, by the coincidence technique, to reduce the contribution of noise and background from a PIPS detector by a factor of ten and to improve its energy resolution. Such a detector system could be a useful tool for quality control of low-level low-energy pure beta-emitters such as ^{63}Ni from environmental samples.

11.6.4 Alpha particle spectrometry

Common radioisotope sources of alpha particles are listed in Table 11.6.

Alpha-emitters, except for the first one in Table 11.6, are members of radioactive decay chains. Decay chains are classified into four groups according to their mass numbers. They are Th-series whose mass numbers are 4N (N is integer),the U-series of 4N + 2, the Ac-series of 4N + 3,and the Np-series of 4N + 1. A Np-series does not exist naturally because the half-life of its longest-lived member is three orders of magnitude shorter than the age of the universe. In three natural decay chains activities of each parent and succeeding daughter nuclides are equal. This condition is called radioactive equilibrium. Because the half-lives of the parent nuclides, ^{232}Th, ^{238}U and ^{235}U are much longer than those of their daughter nuclides.

TABLE 11.6

COMMON ALPHA-EMITTERS

Nuclide	Half-life	Alpha energy (% Branching[a])
^{147}Sm	1.07×11^{11} years	2.232 (100)
^{212}Bi	60.55 months	6.051 (72), 6.090 (28)
^{210}Po	138.38 days	5.304 (100)
^{212}Po	0.296 μs	8.784 (100)
^{220}Rn	55.6 s	6.288 (99.93)
^{222}Rn	3.824 days	5.490 (99.9)
^{226}Ra	1600 years	4.602 (5.5), 4.784 (49.5)
^{232}Th	1.405×10^{10} years	3.954 (23), 4.013 (77)
^{234}U	2.45×10^5 years	4.723 (27.5), 4.775 (72.5)
^{235}U	7.038×10^8 years	4.368 (12.3), 4.400 (57)
^{238}U	4.468×10^9 years	4.150 (23), 4.197 (77)
^{239}Pu	2.413×10^4 years	5.105 (11.5), 5.144 (15.1) 5.157 (73.3)
^{240}Pu	6570 years	5.124 (26.4), 5.168 (73.5)
^{241}Am	432 years	5.443 (13.1), 5.486 (85.2)
^{244}Cm	18.1 years	5.763 (23.6), 5.805 (76.4)

[a] Total intensity is normalized as 100%.

Alpha particles emitted from nuclides which decay to a single level are observed as mono-energy particles. On transitions given the branching ratio in Table 11.6, multiple alpha energies are observed. Such a fine structure in the alpha spectrum comes about because an alpha-emitter may decay to any one of several discrete energy levels of its daughter.

The detection of alpha particles is based on the physics of the processes which take place when the particles pass through matter. During this passage the alpha particle loses its energy by excitation and ionization of the atoms. The energy loss per unit path length is called the stopping power $(-dE/dx)$ and its unit is MeV mg^{-1} cm^2 or eV/(10^{15} atoms cm^{-2}). The stopping power at an energy above 1 MeV is inversely proportional to the alpha-particle energy, while for the energies less than 1 MeV the stopping power is nearly proportional to the energy.

Stopping-powers of various elements are given by Ziegler [62] and Northcliff and Schilling [63]. Stopping power at energies above 1 MeV is inversely proportional to the alpha energy. At the energy-range of less than 1 MeV, the stopping power is nearly proportional to the energy. Because the velocity of alpha particles is low compared with orbital electrons, so alpha particles capture electrons and their effective charges for ionization decrease. When alpha particles pass through matter, they make small-angle scattering. Therefore an asymmetric line spectrum which has its tail extending to the lower-energy side is observed after penetrating relatively thick foils, because of asymmetry in their trajectory path.

The source thickness has an important effect on the observed alpha-particle spectrum. As alpha particles have a relatively large stopping power, the observed spectra for thick sources show some degradations caused by self-absorption. For high resolution spectrometry, thin alpha sources or samples are required. They are usually prepared by electro-deposition or vacuum evaporation. With a source-thickness less than 10 μg/cm^2, no effects owing to self-absorption are observed. For such thin sources, a Gaussian-shape line spectrum whose width is limited by the detector energy resolution is observed for a mono-energetic alpha source. When there is an absorber between an alpha source and a detector the observed energy is reduced by the energy loss in the absorber. Absorbers are the entrance window of a detector, the covering material which prevents contamination of a source, the air, and so on. In the case of a thin source, prepared by chemical separation, the emitted alpha particles are observed as line spectra by spectrometers having high energy resolution. The intensity of each alpha-emitter is easily estimated from the area of the corresponding peak. Even if the peak is superimposed on the tail of another peak, the peak area can be calculated from the shape of the line spectrum.

An excitation is found for the plutonium isotopes, ^{239}Pu and ^{240}Pu. These are used for the estimation of a burn-up of nuclear fuel. As the energy difference of these alpha-emitters is only 10 keV, the alpha-particle spectrum is observed as an overlapped single peak. However, when a Si detector is used, which has an energy resolution of less than 10 keV (FWHM), the overlapped peaks can be analysed by the least-squares fitting technique.

Alpha-particle spectrometry is usually performed using Si detectors, which are especially useful for thin and small-area alpha sources. Two types of Si detectors are commonly used; the surface-barrier type and the ion-implanted PN-junction type. As the thickness of the entrance window of these detectors is less than 80 μg/cm^2 equivalent of Au, the energy loss in these layers is negligible for alpha-particle spectrometry.

As an alternative to Si detectors, a Frich grid ionization chamber is sometimes used as a spectrometer for alpha particles. This ionization chamber has a grid between a cathode and anodes and a sample is put on the cathode electrode. Electrons and ions, which are generated between the grid and the cathode by ionization, drift towards the anode or the cathode, and a signal pulse is obtained. The pulse height obtained from the cathode depends on the emission angle of alpha particles. Only the drift of electrons is observed, owing to their drift velocity being 1000 times greater than that of ions. However, the height of the anode pulse is proportional to the alpha energy. The advantage of this counter is that the areas of samples can be made larger than with Si detectors. A commercially available counter of this type has an area larger than 1000 cm^2. The energy resolution of this counter is 40–50 keV (FWHM) for alpha particles. Several other types of spectrometers can be used in some specific applications. for example, an organic liquid scintillation counter is useful for detection of extremely low-level alpha activities. Alpha-emitters, chemically separated from samples, are mixed into the scintillator. The geometrical efficiency is then 100%, and a relatively large amount of source can be introduced into the scintillator. The main drawbacks of the counter are its poor energy resolution and its relatively low light output in excitation by alpha particles compared to that by electrons.

Track detectors are also often used for alpha detection. The length of tracks produced by alpha particles is measured with a microscope after appropriate chemical etching. The energy spectrum of alpha particles is obtained from the distribution of their trace lengths. Advantages of this detector are its high sensitivity, good discrimination against β- and γ-ray backgrounds and the acquisition of alpha-emitter distribution in samples. Its main drawbacks are a poor energy resolution and the time-consuming processing of the microscope readings.

It should be mentioned that the energy of alpha radiation is one of the most important characteristics of radionuclide sources. Knowledge of the alpha-particle energy is necessary for the determination of other major characteristics of external radiation of alpha sources, such as the flux and energy flux density, and the absorbed dose-rate. Information on the alpha radiation energy is also used for calibrating semiconductor alpha spectrometers. The results of accurate measurements of alpha-particle energies play an important role in the evolution of the atomic-mass scale and comprise nearly 60% of the input data for atoms with $A > 200$, and are deciding factors in the design of precision spectrometers for high-precision energy measurements of alpha particles from radionuclide sources with the minimum attainable uncertainties. A very precise measurement of alpha-particle's energies can be achieved by the measurement of the alpha particle time-of-flight. Such a spectrometer has been recently described by Frolov [64].

When applied to environmental samples, the procedures schematically presented in Fig. 11.4 should be followed. A special problem is the assay of alpha-particle emitters in water samples. There are several procedures in the literature for assaying alpha-particle-emitting radionuclides in natural waters. However, few of them are well suited for low-level analysis since they require the handling of large water samples and the application of concentration techniques. Typical water samples do not contain sufficient amounts of some important nuclides for a precise or reasonably rapid measurement. The most common concentration technique is the co-precipitation of the heavy elements by the addition of a carrier. Some of the best-known methods are coprecipitation with iron as the hydroxide and with calcium and strontium as their oxalates [65]. However, these

methods present some disadvantages. These include the transportation of large volumes of water (several, to hundreds of litres) to the laboratory, the isotopic exchange of tracers, the use of large containers and laboratory ware, and the difficulty of recovering the precipitate. As a consequence of these difficulties, interest in studying in situ concentration techniques with appropriate adsorbents has increased. These methods eliminate the need for preservation, storage, evaporation or co-precipitation of large water samples. One such approach has been described in detail by Crespo et al. [66]. According to the authors, MnO_2 and Al_2O_3 can be used as absorbents in the assay of the low levels of alpha-emitters in the waters. This technology requires more development work before it can become routine.

Another problem of importance to safeguards and reactor fuel technology is the measurement of the relative abundance of plutonium isotopes. In addition to γ- and X-ray spectrometry [67], alpha-particle spectrometry has also been used [68–70]. This usually results in a complex alpha spectrum, with difficulties in obtaining correct amplitudes for overlapping peaks which tail towards lower energies. The ^{239}Pu and ^{240}Pu peaks overlap when alpha-particle energies are measured using Si detectors. Some sort of deconvolution procedure is required in this case (see e.g. [68]).

The radiation dose to man, owing to radon and its decay products in the environment, amounts to more than 50% of the total radiation dose to man from natural radiation sources. Recently, the risk of developing lung cancer from exposure to inhaled radon
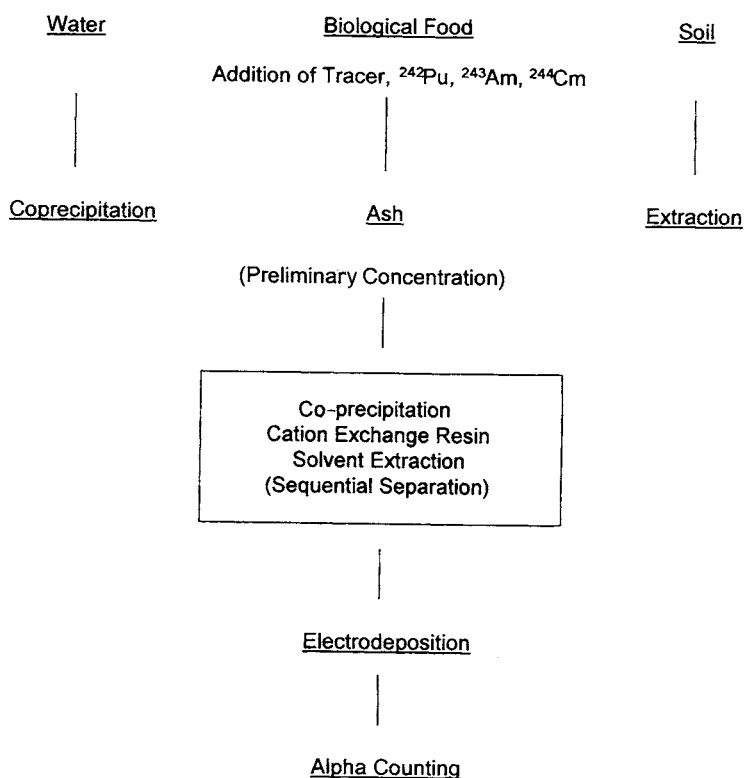
Fig. 11.4. Schematic procedure for sample treatment for alpha counting.

daughters has been attracting considerable attention. Thus, it is very significant to measure alpha-activity levels in the air.

In this chapter we shall describe briefly a filter-sampling method used to assay the gross alpha-activity concentration of radon daughters in the air (indoor air) by means of gross alpha-particle counting with ZnS(Ag) scintillation counters. The data from such measurements may be applied as the natural background level in monitoring man-made alpha-active nuclides in the air.

An air-sampler is used to collect natural radionuclides onto a filter paper by passing air through, because some nuclides of radon progeny (RaA $=^{218}$Po, RaB $=^{214}$Pb, RaC $=^{214}$Bi and RaC$' =^{214}$Po etc.) tend to attach to aerosol particulates floating in the air.

If we suppose that only one radionuclide is attached to the surface of a filter paper, the rate of change with time in the total number of the radioactive atoms (nuclei) on the filter paper can be written

$$\frac{dN}{dT} = \xi qn - \lambda N \tag{6}$$

where t is sampling time; N, total number of radioactive atoms (nuclei) on filter paper at time t; ξ, flow rate of sample air (constant); n, concentration of radioactive atoms (nuclei) in the air (assumed to be constant); λ, decay constant of the radionuclide; ξqn, rate of collection; λN, rate of decay.

The solution of Eq. (6) for the initial condition $N = 0$ at $t = 0$ is

$$N = \frac{\xi qn}{\lambda}(1 - e^{-\lambda t}) \tag{7}$$

The radioactivity A is given by λN, or

$$A = \xi qn(1 - e^{-\lambda t}) \tag{8}$$

The counting rate, R, of alpha particles emitted from the filter paper at time t is related to the radioactivity A,

$$R = \varepsilon\zeta A \tag{9}$$

where ε is counting efficiency of alpha ray counter; ζ, emerging efficiency of filter paper.

From Eqs. (8) and (9) the concentration, n, of the alpha-emitting nuclide in the air is obtained:

$$n = \frac{R}{\varepsilon\zeta\xi q(1 - e^{-\lambda t})} \tag{10}$$

The alpha-activity concentration Q in the air is given by λn, or

$$Q = \frac{\lambda R}{\varepsilon\zeta\xi q(1 - e^{-\lambda t})} = \frac{R}{\varepsilon\zeta\xi q}\frac{0.693}{T}\frac{1}{1 - (1/2)^{t/T}} \tag{11}$$

where $T(= 0.693/\lambda)$ represents the (alpha-decay) half-life of the radionuclide. For long-lived radionuclides such as ^{328}U, ^{239}Pu and ^{226}Ra , Eqs. (7), (8) and (11) become

$$N = \xi qnt \tag{12}$$

$$A = \lambda \xi q n t \tag{13}$$

$$Q = \frac{R}{\varepsilon \zeta \xi q t} \tag{14}$$

respectively.

11.6.5 Liquid scintillation measurement method

Liquid scintillation counting (LSC) has been accepted as the generally preferred method of counting weak beta-emitters and is useful, to a lesser extent, for alpha- and gamma-emitters. The counting sample consists of three components, the radioactive material, an organic solvent or solvent mixture, and one or more organic phosphors. A particle or radiation emitted by the sample material is absorbed in, and its energy transferred to, the solvent and then to the phosphor which emits a scintillation of light-photons. These photons are absorbed by the photocathode of a photomultiplier tube which converts them into an electronic pulse. The pulse, after suitable amplification, is registered as a count corresponding to the emission of the particle or radiation [71]. The technique has the following characteristics:

- self-absorption is usually negligible;
- there is no absorption of radiation by air or a detector's window between the radioactive sample and the sensitive region of the detector;
- there is no radiation scattering prior to incidence upon the detector;
- 4π-counting is performed, because the radioactive material is completely surrounded by the liquid scintillator.

Based on the above merits, the liquid scintillation measurement is extremely sensitive to the low-level radioactivity existing in the environment and food. Since the radioactive sample material from most methods of sample preparation is in intimate contact, or in actual solution, with the phosphor, the detection of emitted particles or radiation is highly efficient and may even approach 100%. Problems of self-absorption of the emissions are thus absent, or considerably smaller than those associated with planchette counting of solid samples. This is of particular importance for the measurement of low energy beta-emitters such as tritium and carbon-14. On the other hand, the measurement method has intrinsic drawbacks such as quenching and chemiluminescence.

Currently, the liquid scintillation counter has been employed not only for the measurement of low-energy β-emitters, but also for pure β-, β-γ-, and α-emitters and further Cherenkov radiation. The liquid scintillator consists mainly of organic solvent and fluorescent material (i.e. solute), and sometimes a surfactant or other material is added to the solution. The characteristics of the liquid scintillator depend mostly on the sort and amount of these chemicals. The liquid scintillator plays the role of an energy transducer, converting radiation energy into photons. The organic solvent which comprises most of the liquid scintillator should satisfy the following conditions [71]:

- the energy should transfer efficiently in the process of luminescence;
- the absorption spectrum of the solvent should never overlap an emission spectrum of a solute;

TABLE 11.7

TYPICAL SOLVENTS USED FOR LIQUID SCINTILLATOR

Solvent	Molecular weight	Solidifying point (°C)	Absorption spectrum (A) λ max[a]	Emission spectrum (A)		Relative pulse height
				λ max	λ mean[b]	
Toluene	92.13	−95	2.620	2.870	2.840	1.00
Xylene	106.16	−20	2.660	2.890	2.880	1.09
Pseudo-cumene	120	−60.5	2.690	2.930	2.900	−
Dioxane	88.1	12	1.880	2.470	−	0.65

[a] Wavelength giving maximum value.
[b] Wavelength giving mean value.

- the radioactive sample and solute must be able to be incorporated with the solvent;
- the solvent must be of high purity.

In the past, many kinds of chemicals were employed as solvents, but nowadays only a few typical solvents are being used /including toluene, xylene, pseudocumene, and drioxane.

Characteristics of typical solvents used for liquid scintillators are shown in Table 11.7.

The solute is classified into a primary solute and a secondary one. The former is the main fluorescent material, and the latter serves as a wavelength shifter which give rise to an emission spectrum having a longer wavelength (see Table 11.8). The sample to be measured is easily prepared by incorporating a radioactive sample into the liquid scintillator such as a xylene-based-, toluene-based-, or emulsive scintillator. The xylene (or toluene)-based scintillator is appropriate for hydrophobic samples, to form a homogeneous solution which provides efficient energy transfer and light-counting efficiency.

The simplest mixture consists of a primary scintillator and a secondary scintillator dissolved in a primary solvent. The primary solvent converts the kinetic energy of radiations into excitation energy. The primary solvents are usually aromatic hydrocarbons, and

TABLE 11.8

TYPICAL SOLUTES USED FOR LIQUID SCINTILLATOR

Solute	Absorption spectrum λ max[a]	Emission spectrum		Optimum concentration (g/l)	Decay time of scintillation
		λ max	λ mean[b]		
Primary solute PPO	3.030	3.640	3.703	4–7	1.6
Secondary solute DMPOPOP	3.630	4.290	4.273	0.2–0.5	1.5
bis-MSB	3.470	4.120	4.219	ca. 0.5	1.3

[a] Wavelength giving maximum value.
[b] Wavelength giving mean value.

therefore only non-polar radioactive materials can be dissolved in them. The most widely used primary solvent is toluene. Others are *p*-, and *m*-, and mixed xylenes. Pseudocumene (1,2,4-trimethylbenzene) is becoming a popular solvent for new, commercially produced scintillation cocktails. It offers the highest energy conversion efficiency of the known solvents, and has fewer restrictions on shipping and storage as a combustible liquid because of its high flash-point, low volatility, and lower toxicity.

Owing to the presence of organic solvent, the mechanism of luminescence for the liquid scintillator is much more complicated than that for a crystal scintillator. The energy transfers successively to generate, finally, the luminescence as follows:

- excitation of a solvent molecule by the absorption of radiation energy;
- solvent–solvent energy migration;
- solvent–solute energy transfer;
- Luminescence from the solute molecule.

Since the solvent molecules have a majority in the liquid scintillator, they are initially excited by the radiation energy. The π-electrons of the solvent molecule plays an important role in the process of energy-transfer owing to their active mobility.

When the solute absorbs the excitation energy liberated from the solvent, the solute molecules in the ground state are excited up to the excited electron level or a vibrational one. Then, the following processes compete with one another as intramolecular behaviours of the solute:

- internal conversion of the molecule;
- fluorescence emission;
- intersystem crossing;
- phosphorescence emission.

The process which is most important among these phenomena, and the fluorescence produced therein, is detected as a signal in an electric circuit.

The quenching is a phenomenon caused by the energy loss in the process of energy-transfer inside the liquid scintillator. It should be taken into account whenever the liquid scintillation measurement is performed. Owing to the existence of a quencher, which means the material giving rise to the quenching, the counting efficiency finally decreases as follows:

- energy is partly lost during the energy transfer;
- the number of photons emitted from the solute decreases, and/or some of the photons are wastefully absorbed in solution;
- the height of the electric pulse created by a photomultiplier tube becomes lower;
- a pulse-height distribution shifts toward a lower pulse-height;
- the number of pulses present within a countable region decreases to lead to the lowering of the counting efficiency;
- sample-preparation techniques involve the chemical separation of the specific chemical forms of a radionuclide, and the preparation of the counting-sample by mixing the separated nuclides with the proper liquid scintillator.

A large number of recipes for scintillation mixtures has been published, but for many purposes a few simple ones will suffice. The properties of a suitable mixture are as follows:

- it should generally be clear, colourless and homogeneous after the addition of the radioactive sample, although in certain cases suspensions or gels may be satisfactory;
- it should quench as little as possible – this is particularly important when measuring radioisotopes such as tritium, whose beta particles are of low energy;
- it should not be expensive;
- its constituents should be stable. Some scintillators are known to be unstable when exposed to light, and other materials may deteriorate on storage, with the formation of impurities which cause quenching or chemiluminescence. Scintillation mixtures should be stored in dark bottles.

Some of the radionuclides commonly distributed in environmental samples include ^{3}H, ^{14}C, ^{6}Co, ^{89}Sr and ^{90}Sr. Tritium and ^{14}C emit low-energy betas which are efficiently counted by LSC. Here we present some details of sample-preparation for LSC. Aqueous samples containing tritium are distilled to eliminate impurities, and mixed with an emulsifier scintillator with the proportion of 40–50 (v/v) water. Tritium in biological samples exists as tissue free water tritium (TFWT) and organically bound tritium (OBT). Water containing TFWT is obtained by vacuum distillation (lyophilization) or azeotropic distillation using organic solvents such as toluene and benzene. The OBT is converted into HTO for counting by combusting dried samples.

The tritium concentration in the natural environment is less than 10 Bq/l and sometimes less than 1 Bq/l. Only low-background type LSCs detect these low tritium levels. To measure tritium below 10 Bq/l with good accuracy, using the conventional type LSCs, it is necessary to enrich the tritium in a large water sample by electrolysis. This procedure can increase the tritium concentration 20–30-fold.

Carbon-14 exists mostly as carbon dioxide (CO_2) and as organic compounds in the environment. Carbon dioxide in the atmosphere or in the effluent gas from a combustion system for biological samples is first absorbed in alkaline solutions such as aqueous NaOH or NH_4OH. If necessary, calcium chloride or barium chloride is added to the alkaline solution to precipitate $CaCO_3$ or $BaCO_3$, which are then purified and stored in a sealed bottle for future analysis. The alkaline solution containing carbon dioxide or carbonate is acidified by titrating with a strong acid solution to generate carbon dioxide. The carbon dioxide gas is then absorbed in a solution of organic amine absorber and liquid scintillator mixture (1:1).

The Procedure for ^{60}Co measurement is as follows. After adding the cobalt salt as carrier to sea-water, Co-60 is first precipitated as cobalt hydroxide in alkaline solution. The precipitate is separated from the sea-water and dissolved in HCl solution. Co-60 is separated and recovered in the effluent from an anion exchange column. The effluent containing Co-60 is evaporated to dryness. The residue is redissolved in dil. HCl solution and transferred into a counting vial. The emulsifier scintillator is added into the vial and mixed well. The counting sample is counted by means of a low background LSC. This technique can be applied to Co-60 in biological samples by using an ashing procedure beforehand.

Strontium isotopes can be measured by utilizing the fact that high-energy beta rays in aqueous solution emit Cerenkov photons which can be counted by photomultipliers in a LSC without any phosphors.

Strontium radioisotopes in a sea-water sample are separated and purified by precipita-

tion of strontium carbonate and subsequently strontium oxalate precipitate followed by the cation exchange. Yttrium-90, the daughter nuclide of ^{90}Sr, is scavenged from the solution by Fe(OH)$_3$ co-precipitation. The acidified aqueous solution containing strontium isotopes is transferred into a counting vial and counted after the total volume of the solution is adjusted by adding distilled water.

By setting the lower level of the discriminator properly, the LSC can count only Cerenkov light signals produced by 1.49 MeV beta rays of ^{89}Sr, without counting the 0.546 MeV beta rays of ^{90}Sr. ^{90}Sr is determined by counting Cerenkov photons generated by 2.28 MeV beta rays of ^{90}Y which grows into the solution.

11.6.6 Radiochemical analysis

11.6.6.1 Introduction
Considerable attention has focused on long-lived artificial radionuclides such as ^{90}Sr and ^{137}Cs released into natural environments. Their inventories began to grow as a result of nuclear weapon testing programs since 1950 and the nuclear power reactor accident of Chernobyl in 1986. The new sources contaminating the environment with radionuclides are thought recently to be liquid effluents from nuclear power stations and nuclear fuel reprocessing facilities. The long-lived fission products and their characteristics are given in Table 11.9 [72–77].

It is not practicable to present here all the methods used; we outline only the reliable methods used for ^{89}Sr and ^{90}Sr, for strontium, tritium and for actinides (for more details see [45]).

11.6.6.2 Analysis of strontium

Strontium-90 is one of the most important fission products, because of its relatively high yield (about 6%), long physical half-life (29 years), and its uptake and retention in biological systems. For assessing the integrated exposure to large populations, not only the direct measurements of biological materials must be done but also the monitoring of the transportation of the nuclide in the environment, e.g., in oceans and streams.

The assay of low-level strontium-90 in biological and radiotoxicological samples requires time-consuming and laborious techniques because both the nuclide ^{90}Sr and its daughter ^{90}Y are pure beta-ray emitters. Therefore, the radio-strontium must be completely separated from other radionuclides prior to the beta-ray counting. The most commonly used method for separating strontium is by nitrate precipitation. With some modifications this method can be applied to all kinds of environmental samples and foods. The chemical yield varies according to the type of material. The use of ^{85}Sr tracer to determine chemical yield is a general practice. When determining the yield in this manner, it is important that the tracer is pure ^{85}Sr, i.e., free from ^{89}Sr and ^{90}Sr. Although the method is time-consuming, it is reliable and safe. Rapid methods for ^{90}Sr analysis exist, and it has been shown that they can be used after short-lived nuclides have decayed. In fresh fallout situations, the nitrate precipitation method has been shown to be more reliable. Also, during periods of fresh fallout, the amount of ^{89}Sr is of interest and the rapid methods can only analyse for ^{90}Sr. In the case of higher contamination with ^{90}Sr, the daughter ^{90}Y can be separated without waiting for equilibrium. Within 10 h the activity concentration of ^{90}Y will be

TABLE 11.9

LONG-LIVED FISSION PRODUCTS

Nuclides	Decay mode	Half-life (years)
^{79}Se	β^-	6.5×10^4
^{95}Kr	β^- (γ)	10.7
^{97}Rb	β^-	4.8×10^{10}
^{90}Sr	β^-	28.8
^{93}Zr	β^-	1.5×10^6
^{99}Tc	β^-	2.14×10^5
^{106}Ru	β^- (γ)	1.00
^{107}Pd	β^-	6.5×10^6
^{113m}Cd	β^- (γ)	14.6
^{121m}Sn	β^- (γ)	55
^{125}Sb	β^- (γ)	2.7
^{126}Sn	β^- (γ)	1×10^5
^{129}I	β^- (γ)	1.57×10^7
^{133}Cs$(n,\gamma)^{134}$Cs	β^- (γ)	2.06
^{135}Cs	β^-	3×10^6
^{137}Cs	β^- (γ)	30.17
^{147}Pm	β^- (γ)	2.62
^{155}Eu	β^- (γ)	4.9

approximately 10% of the equilibrium value and may be sufficient for a reliable ^{90}Sr analysis [45].

A special application of liquid scintillation counters is in the measurement of Cerenkov radiation produced by beta-emitters with beta energies greater than 260 keV. This application can be used for screening samples for ^{90}Sr [78–86].

An outline of the method used for determination of radio-strontium in various materials by nitrate precipitation is as follows. The ashed material is dissolved in nitric acid in the presence of strontium-and barium carriers. The nitric acid concentration is then increased to precipitate all the strontium and barium (and part of the calcium) as nitrates. After further nitric acid separations, barium chromate and iron hydroxide scavenges are carried out. The subsequent treatment depends somewhat on the circumstances, but the following is normal practice. An yttrium carrier is added to the purified strontium solution and, after a delay of about 14 days for the growth of ^{90}Y, the yttrium is separated, mounted and counted. The storage period for the growth of ^{90}Y can be reduced if sufficient ^{90}Sr is known to be present, and the appropriate growth factor applied. For samples of very low activity, as well as for measurement of ^{89}Sr, strontium is precipitated from the solution remaining after the removal of yttrium, and mounted for counting. In many cases, the determination of the natural inactive strontium content of the material is required so that the strontium chemical yield can be corrected.

For milk, direct application of the nitric acid separation to a solution of the ash usually gives low strontium yields. The calcium, strontium and barium are therefore concentrated by an initial phosphate precipitation. The mixed phosphates are then dissolved in an acid and the general procedure continued from that point.

For cereals, and vegetation generally, the ash is very variable in composition and contains numerous elements other than calcium: a mixture of hydrofluoric and perchloric acids is necessary to decompose and dissolve the ash. After heating to remove the hydrofluoric and most of the perchloric acid, the residue is dissolved in dilute acid and the alkaline earths precipitated as phosphates. For details of the procedure, see IAEA Report No. 295 [45] and references therein. A procedure is given, designed to separate ^{90}Y from a partially purified Sr fraction isolated from various marine samples. It has been used successfully in soil, sediment and ashed biological samples up to 300 g in weight and in fresh-water and sea-water samples, up to 600 litres in volume. ^{90}Sr levels as low as 1 ppm have been measured in these samples.

Several important facts should be kept in mind when planning ^{90}Sr analysis on marine samples. Average sea-water contains 8 ppm of stable Sr, or about 8 mg kg^{-1}, while average carbonate soil has about 1% (10 000 mg kg^{-1}) Sr. Thus even modestly sized samples require some thought in sample-handling and about the chemical yield measurements, especially if stable Sr is to be used for a yield monitor. This procedure has been developed to accommodate a Sr carrier from 20 mg to 4 g. For most routine analysis, however, ^{85}Sr, a gamma-emitter readily measured by gamma spectrometry and free from ^{90}Sr contamination, is the preferred yield monitor. If stable strontium is used for chemical recovery, the strontium content of the original sample has to be determined.

In average sea-water the ^{226}Ra content is about 0.2 dpm/l and ^{238}U (or ^{234}U) is about 2 dpm/l. The uranium usually presents no problem, but a good separation of the final ^{90}Y from Ra and its daughters is important, especially for deep ocean water. During the separation steps, this procedure effectively removes all radionuclides that are chemically similar to yttrium or rare earths from the Sr fraction. And after the establishment of $^{90}Y/^{90}Sr$ radioactive equilibrium, the final purification steps further remove any interfering radionuclides in the growth ^{90}Y fraction before measurement by beta counting.

11.6.6.3 Analysis of tritium

Tritium is measured by liquid scintillation counting of a portion of a distilled sample. Several reagents (such as sodium sulfite and silver iodide) can be added in the distillation to prevent interference by radio-iodine. The allowed concentration of tritium in water for human consumption is relatively high; thus the method presented here is normally adequate for routine determinations. However, if required, lower concentrations of tritium in water can be determined by electrolytic enrichment. The principles of the tritium determination procedure are as follows.

The water sample is distilled to remove non-volatile quenching materials and non-volatile radioactive materials. Prior to distillation, sodium carbonate (Na_2CO_3) and sodium thiosulfate ($Na_2S_2O_3$) are added to the sample. The majority of the constituents that might interfere remain in the residue, together with any radioactive iodide and bicarbonate that might be present. If the tritium content of non-aqueous biological samples is required, the sample can be converted into water by oxidation. The distillation is carried out to dryness to ensure complete transfer of the tritium to the distillate. An aliquot of the distillate is mixed with a scintillation solution in a counting vial. The mixture is cooled and counted in a liquid scintillation spectrometer (coincidence type). In this sample (usually an emulsion) the kinetic energy of the tritium beta particles is partly converted into light-photons. When

certain boundary conditions are satisfied (e.g., simultaneous detection by two or more photomultiplier tubes connected in coincidence, and discrimination of pulses by measurement channels present), these photons are counted as pulses.

Standard tritium- and background-samples are prepared and counted identically to minimize errors produced by ageing of the scintillation medium or by instrumental drift. The counting rate is a measure of the tritium activity concentration. the sensitivity (counting-time, 100 min) is generally of the order of 20–200 Bq l^{-1}. Details of the method are described in [45]. For additional reading, see [83,87–96].

11.6.6.4 Determination of actinides

Actinides in the environment can be classified into two groups: (i), the uranium and thorium series of radionuclides in the natural environment, and. (ii) neptunium, plutonium, americium and curium, which are formed in a nuclear reactor during the neutron-bombardment of uranium through series of neutron-capture and radioactive-decay reactions. The transuranics thus produced have been spread widely in the atmosphere, geosphere, and aquatic environment on earth, as a result of nuclear bomb tests in the atmosphere, and accidental release from nuclear facilities [97]. Most of these radionuclide inventories have deposited in the northern hemisphere following the tests conducted by the United States and the Soviet Union.

In the actinide series, the elements of greatest interest as environmental contaminants are neptunium, plutonium, americium, and curium, because their presence at relatively high concentrations in ecosystems would represent potential health problems [98]. Nuclear data for actinide isotopes are presented in Table 11.10.

As a result of nuclear-bomb tests in the atmosphere, accidental release from nuclear facilities, and the accidental fall of artificial satellite SNAP 9A, plutonium isotopes has been spread widely into atmosphere, geosphere and aquatic environment on the earth from the 1960s. They include ^{241}Pu, ^{240}Pu, ^{239}Pu, and ^{239}Pu [76]. These plutonium isotopes in the geosphere and aquatic environment are incorporated metabolically into plants, and ultimately find their way into man through food chain, and these radionuclides in the atmosphere are also incorporated into man, directly by inhalation. From the standpoint of a dose-assessment, these radionuclides are important because they are mostly alpha-emitting radionuclides and have half-lives of 13–2.4 × 10^4 years. From the standpoint of the safety assessment of the disposal of high-level radioactive waste, it is important to clarify the migration behaviour of plutonium in ground strata. Consequently it is essential to know the plutonium concentrations in food and environmental samples for these studies.

The commonly applied methods for determination of actinides in environmental samples may be classified as follows.

Preparation, by drying, ashing and scavenging
Solubilization and equilibration, by fusion or leaching
Concentration and separation by co-precipitation, ion exchange, or solvent extraction
Electrodeposition and alpha spectrometry

For a description of a procedure for plutonium separation in large volumes of fresh and saline water by manganese dioxide co-precipitation see the paper by Wong et al. [99].

Among ion exchange separation methods for transuranics, strong base anion exchange

TABLE 11.10

NUCLEAR DATA OF ACTINIDE ISOTOPES

Nuclide	α-Energy (MeV)	Yield (%)	Half-life (years)
^{244}Cm	5.81, 5.76	77, 23	1.81×10
^{242}Cm	6.11, 6.07	74, 26	6.09×10^6
^{243}Am	5.28, 5.23	88, 11	7.37×10^3 tracer
^{241}Am	5.49, 5.44	86, 13	4.32×10^2
^{242}Pu	4.90, 4.86	74, 26	3.76×10^5 tracer
^{240}Pu	5.16, 5.12	76, 24	6.57×10^3
^{239}Pu	5.16, 5.14, 5.10	73, 15, 12	2.41×10^4
^{238}Pu	5.50, 5.46	71, 29	8.77×10
^{238}Pu	5.77, 5.72	69, 31	2.85 tracer
^{237}Np	4.79, 4.77	51, 36	2.14×10^6
^{238}U	4.20, 4.15	77, 23	4.47×10^9
^{235}U	4.40, 4.37	57, 18	7.04×10^5
^{234}U	4.77, 4.72	72, 28	2.45×10^5
^{232}U	5.32, 5.26	69, 31	7.18×10 tracer
^{232}Th	4.02, 3.96	77, 23	1.41×10^{10}
^{230}Th	4.69, 4.62	76, 23	8.03×10^4
^{229}Th	4.90, 4.85	11, 56	7.34×10^3 tracer
^{228}Th	5.42, 5.34	73, 27	1.91

in hydrochloric and nitric acids are important [99–105]. Among solvent extraction reagents for transuranics, thenoyltrifluoroacetone (TTA) and trioctylamine (TOA) are important [100,106].

Each transuranic element has many valences and their behaviours in aqueous solution are very complicated because of disproportionation reactions. As stated above, the ion-exchange and solvent-extraction behaviours of transuranics are dependent on their valence states. Therefore, valence-control is very important in their analysis [98]. Additional references discussing this problem include [107–117].

Procedures have been described for the determination of plutonium and americium in environmental samples by anion exchange (HNO_3). Procedures are also described for the determination of plutonium, americium and their sequential analysis by anion exchange (HNO_3) and TOA extraction [106]. As an illustration we present in Fig. 11.5 the method for the determination of Am in environmental samples, as developed by [118]. The method for the determination of Np in environmental samples developed by Yamamoto et al. [119] is shown in Fig. 11.6.

11.6.7 Rapid methods

The need for rapid methods is apparent in accidents and similar situations. An impulse to the development of rapid methods is also provided by the so-called coordinated research program of IAEA, Vienna. Here we shall present some of the methods reported in a Research coordination meeting on Rapid Methods held in Vienna in 1991.

Sample leachate (HCl)

↓

Fe solvent extraction

(diisopropyl ether)

↓

Anion exchange (HCl) ⟶ Pu fraction

↓

Cation exchange (HCl)

↓

$BiPO_4$ or $Fe(OH)_3$ precipitation

↓

Anion exchange

$(HNO_3\text{-}CH_3OH)$

↓

Anion exchange (HNO_3) ⟶ Am fraction
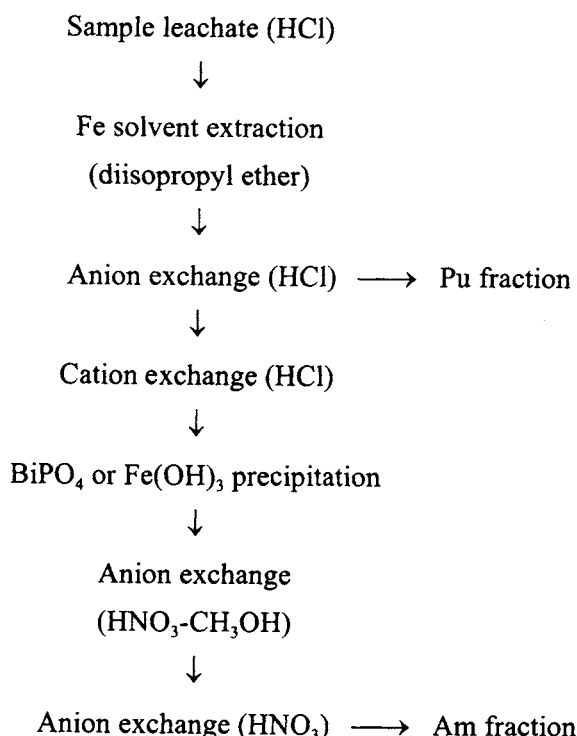
Fig. 11.5. Flowsheet for Pu/Am method (after Livens and Singleton [118]).

11.6.7.1 Radionuclides in bulk food samples

This problem could be approached following the work by Brodzinski and Perkins [120]. They have described a completely portable instrument, operable by one man, which is capable of quantifying the radioactive content of 208-litre drums. Eleven radioisotopes are measured simultaneously in just a few minutes. The assayer uses two measuring techniques: segmented γ-ray spectrometry and neutron-counting. A drum (or other container) to be assayed is placed on a rotating turntable by a self-contained electric hoist. A collimated high-resolution germanium γ-ray spectrometer vertically scans the rotating drum to measure the intensity of γ-rays as a function of the energy emanating from the drum. Most fission and activation products and some transuranic radionuclides emit measurable quantities of monochromatic photons that serve as 'fingerprints' of those radioisotopes. Comparison with emission rate from known standards provides a quantitative measure of the radioactivity from each γ-ray emitter in the drum. The same germanium spectrometer is used to measure the bremsstrahlung radiation from ^{90}Sr. By manipulating the software with the on-board computer, the intensity of the ^{90}Sr bremsstrahlung in the assayed drum is also compared to that of standards, and the ^{90}Sr concentration is quantified. The sensitivity reported for transuranic radionuclides is approximately 1 nCi/g, while that for gamma-emitters is of
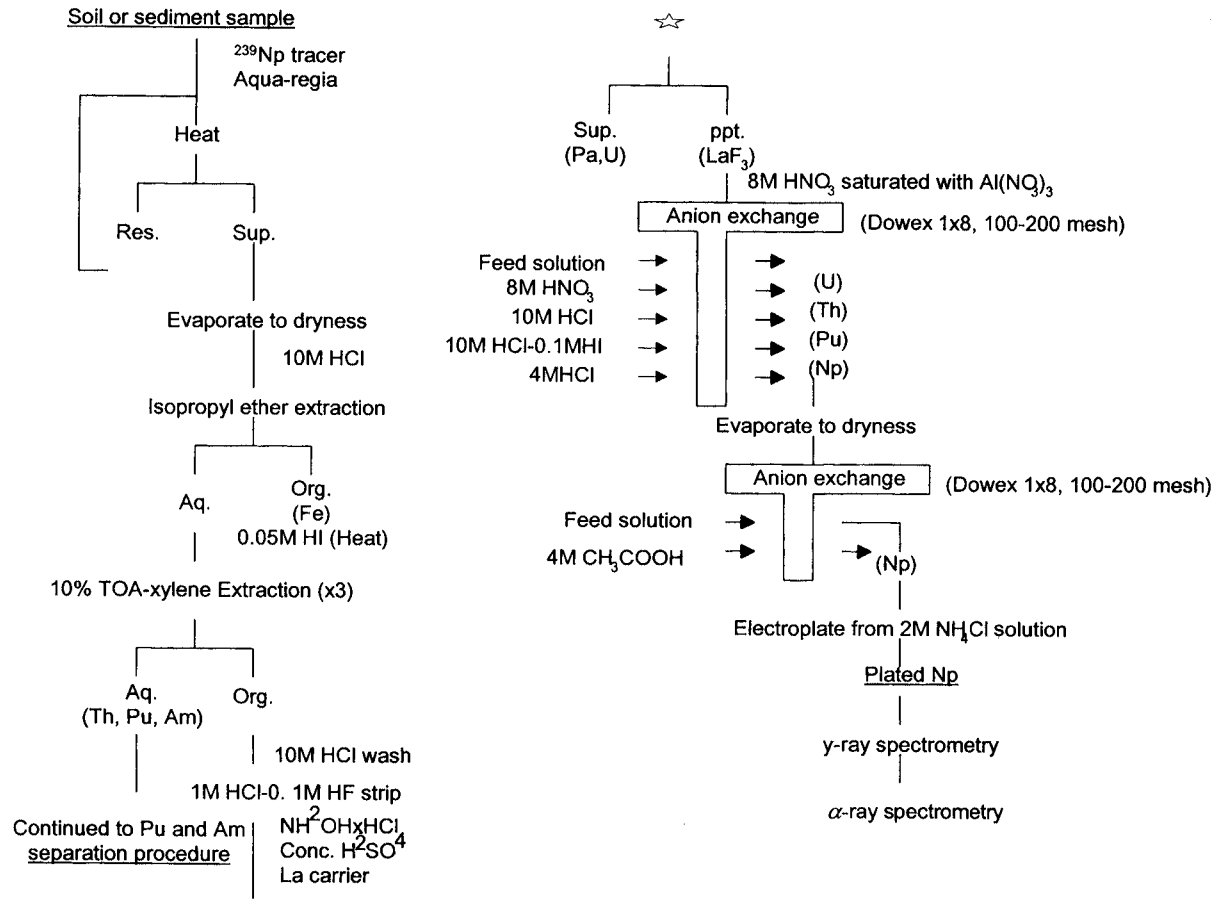
Fig. 11.6. Separation scheme for neptunium in environmental soil or sediment sample (after Yamamoto et al. [119].

the order of 0.1–1 pCi/g. Also, based on the bremsstrahlung radiation measurement, ^{90}Sr can be determined at concentrations of 100 pCi/g.

11.6.7.2 Rapid determination of transuranic elements and plutonium

The rapid methods are based on the fast removal of transuranic elements from interfering materials so that they can be electro-deposited as a group, and measured by alpha-energy analysis. The procedure involves the following basic steps [120].

1. The sample is first brought into solution
2. Radioisotope tracers, including ^{242}Pu, ^{243}Am, and ^{234}Th (if appropriate), are added
3. A small amount of Fe carrier (10 mg) plus sodium sulfite is added to this solution, which is subsequently made basic by addition of ammonium hydroxide, to allow the formation of an iron hydroxide precipitate. This precipitate serves to carry the thorium and the transuranic elements
4. The mixture is centrifuged and the solution discarded: the precipitate is dissolved in dilute hydrochloric acid, then diluted with water. It is then made basic with ammonium hydroxide, which results in a second iron hydroxide precipitate forming
5. Following centrifuging and discarding of the solution, the precipitate is dissolved in dilute hydrochloric acid, diluted with water, and a small amount of sodium sulfite is added to maintain the transuranic elements in their lower valence states
6. Small amounts of calcium and oxalic acid are then added and the pH is adjusted to approximately 3 to allow formation of an oxalate precipitate. The iron forms a very soluble oxalate and thus remains in solution. This, and two subsequent oxalate precipitations, serve to remove any remaining iron
7. The final oxalate precipitate is then dissolved in a small amount of sulfuric acid (0.5 ml of conc. H_2SO_4), and the pH adjusted using dilute ammonium hydroxide
8. The solution is then placed in an electro-deposition cell, where the transuranic elements are electro-deposited on a 1 cm^2 area of a 2.5-cm-diameter stainless steel disc
9. Electro-deposition is conducted for a 1-h period at a current of 1 A
10. Immediately before turning off the current, 1 ml of concentrated NH_4OH is added to the cell and the electro-deposition continued for an additional minute. the current is then turned off, the solution discarded, and the electrode washed with water, then ethanol, and air-dried. Following alpha-energy analysis, the radiochemical yield, as determined from the radioisotope tracer content and the concentrations of the radionuclides of interest, are calculated

Samples with a large amount of iron, such as soil extracts or vegetation ash, may require partial removal of iron prior to initiation of this procedure [120].

11.6.7.3 Rapid determination of ^{90}Sr

Up to now, two different approaches have been used successfully in fast radiochemical separation procedures for the determination of strontium-90 in environmental samples.

These are for investigations dealing with the extraction of yttrium-90 and those which tailor precipitation methods for the strontium-90 separation to the needs of special sample-types.

We mention only some of these. Vajda et al. [121] have described a simple and rapid method for the separation and determination of total radio-strontium in soil by using a crown ether. The method consists of three basic steps: oxalate precipitation to remove bulk potassium, chromatographic separation of strontium from most inactive and radioactive interferences, utilizing a crown ether, and oxalate precipitation of strontium to evaluate the chemical yield. Radio-strontium is then determined by liquid scintillation counting of the dissolved precipitate. When 10-g samples of soil are used, the sensitivity of the method is about 10 Bq/kg. The chemical yield is about 80%. The separation and determination of radio-strontium can be carried out in about 8 h.

Another method for ^{90}Sr determination in food and environmental samples has been described in [122]. It is based on the use of a tributyl solidus (twice) phosphate for extraction of ^{90}Y, the daughter of ^{90}Sr. The method is shown to be sensitive to 0.2 Bq kg of dry grass and milk powder and 2 Bq per of soil.

In the case of a nuclear accident, most of the radioisotopes in the environment and food can be assayed reliably and quickly by gamma spectroscopy. There is a problem with some important isotopes which are pure beta- or alpha-emitters and which cannot be identified directly by gamma spectroscopy. The activity of the isotopes of the strontium group ^{89}Sr, ^{90}Sr and ^{91}Y after a 3-year reactor-fuel cycle can reach about 8% of the total in-core activity, and one of them, ^{90}Sr(Y) is important for long-term health consequences.

It has been shown [123] that the Ba/Sr reactor-core ratio can be used for estimation of the upper limit of the strontium activity in the fallout immediately after an accident. The Cs/Sr ratio can be used for estimation of the strontium fallout in the late post-accident period.

11.6.8 Non-radiometric methods

There is a variety of situations in which it is better to determine the concentration of a radionuclide by a mass measurement rather than by measuring the activity present. This approach is possible using a wide range of instrumental methods of non-radiometric elemental analysis. Analytical measurements can be performed also by element-specific chemical methods, some of which are extremely sensitive.

The most important criterion for selecting an analytical method is whether the technique is sufficiently sensitive to measure the amount of radionuclide present in the sample. This is a very different problem when considered from the viewpoint of analytical chemists who use radiometric methods and those who use non-radiometric methods. Limits of detection in radiometric methods can be as low as 10^{-4} Bq, although ~ 1 mBq is a more generally attainable detection limit. For non-radiometric methods, the detection limit is expressed in terms of mass, and the relationship between radiometric and non-radiometric limits of detection will depend upon the half-life of the radionuclide of interest.

Table 11.11 shows the mass corresponding to 1 mBq for a number of important radionuclides. Although 1 mBq of ^{232}Th is readily measured by a number of non-radiometric methods, 1 mBq of ^{137}Cs could only be detected by the most sensitive methods and is probably best determined radiometrically.

TABLE 11.11

THE MASS OF 1 mBq FOR A SELECTION OF RADIONUCLIDES WITH A VARIETY OF HALF-LIVES [124]

Radionuclide	Half-life (years)	Mass of 1 mBq (g)
^{232}Th	1.4×10^{10}	2.5×10^{-7}
^{238}U	4.5×10^{9}	8.1×10^{-8}
^{129}I	1.7×10^{7}	1.6×10^{-10}
^{99}Tc	2.1×10^{5}	1.6×10^{-12}
^{239}Pu	2.4×10^{4}	4.3×10^{-13}
^{14}C	5.8×10^{3}	6.1×10^{-15}
^{137}Cs	30	3.1×10^{-16}

Non-radiometric methods offer a variety of features, and their use may be favoured for reasons other than improved sensitivity or isotopic selectivity. They can, in some instances, be used to perform analyses with less sample-preparation and greater speed or sample throughput, and allow remote analysis or provide elemental or isotopic maps or depth profiles [124].

The instrumental methods of elemental analysis can be conveniently grouped as below.

1. Methods based on X-ray fluorescence analysis

   X-ray fluorescence analysis (XRF)
   Total reflectance X-ray fluorescence analysis (TXRF)
   Electron-microprobe analysis (EMA)
   Particle-induced X-ray emission (PIXE)
   Synchrotron-radiation induced X-ray emission (SRIXE)

2. Methods based on ultraviolet or visible spectroscopy

   Atomic absorption spectroscopy (AAS)
   Graphite furnace AAS (GFAAS)
   Atomic fluorescence spectroscopy (AFS)
   Inductively-coupled-plasma optical-emission spectroscopy (ICPO–ES)
   Glow-discharge optical-emission spectroscopy (GC–OES)
   Laser-excited resonance ionization spectroscopy (LERIS)
   Laser-excited atomic-fluorescence spectroscopy (LEAFS)
   Laser-induced-breakdown spectroscopy (LIBS)
   Laser-induced photoacoustic spectroscopy (LIPAS)
   Resonance-ionization spectroscopy (RIS)

3. Methods based on mass spectrometry

   Spark-source mass spectrometry (SSMS)
   Glow-discharge mass spectrometry (GDMS)
   Inductively coupled-plasma mass spectrometry (ICP–MS)
   Electro-thermal vaporization–ICP–MS (ETV–ICP–MS)
   Thermal-ionization mass spectrometry (TIMS)

Accelerator mass spectrometry (AMS)
Secondary-ion mass spectrometry (SIMS)
Secondary neutral-mass spectrometry (SNMS)
Laser mass spectrometry (LMS)
Resonance-ionization mass spectrometry (RIMS)
Sputter-initiated resonance-ionization spectroscopy (SIRIS)
Laser-ablation resonance-ionization spectroscopy (LARIS)

McMahon [124] has reported on the intercomparison of non-radiometric methods for the measurement of low levels of radionuclides. He has classified the above analytical techniques according to the amount of isotopic information and the amount of sample required. The conclusions are presented in Table 11.12.

11.6.8.1 Methods based on X-ray spectrometry

The electronic transitions which give rise to X-ray emission spectra involve core electrons and are therefore relatively insensitive to the chemical and physical form of the determinant [125]. As a result analyses can be performed with a minimum of sample preparation, directly on materials in the condensed phase. This insensitivity to the sample matrix applies to the wavelength of the emitted X-rays, not to their intensities and as quantitation is based on intensity measurement closely matched standards are required: X-ray emission spectra can be excited by primary X-rays in a fluorescence experiment or by changed particles via collisional excitation. The cross-sections for excitation of X-ray emission are rather low, and this is combined with the low efficiency of collection, collimation, diffraction and detection of the emitted X-rays. This low overall efficiency leads to a relatively low sensitivity in some cases, and is compounded by high backgrounds either from scattered primary radiation in a fluorescence experiment, or from bremsstrahlung in the charged-particle-excitation methods. Methods based on X-ray spectrometry do not provide isotopic information about the sample. Nonetheless, a number of radio analytical problems can be solved by methods based on X-ray spectrometry.

The following instrumental methods of elemental analysis are based on X-ray spectrometry.

TABLE 11.12

ANALYTICAL TECHNIQUES CLASSIFIED BY AMOUNT OF ISOTOPIC INFORMATION AND AMOUNT OF SAMPLE REQUIRED [124]

	Bulk samples	Small samples profiling	Imaging and depth
No isotopic information	ICP–OES	XRF, GFAAS, LEAFS, TXRF	PIXE, SRIXE
Minor isotope determination	ICP–MS, GDMS, SSMS	ETV–ICP–MS	SIMS, SNMS, LMS, SIRIS
Trace isotope determination		TIMS, RIMS, AMS	

X-ray fluorescence analysis (XRF)
Total reflectance X-ray fluorescence analysis (TXRF)
Electron microprobe analysis (EMA)
Particle-induced X-ray emission (PIXE)
Synchrotron radiation-induced X-ray emission (SRIXE)

XRF is the simplest of these methods. It permits bulk analysis of solid or liquid samples with detection limits of approximately 0.1 μg. The method can thus only compete with radiometric methods for the longest-lived of radionuclides. It has approximately the same sensitivity for ^{232}Th as alpha spectrometry but has the advantage that little sample preparation is required and that analysis is rapid and easily automated. XRF would be the method of choice for measurement of airborne thorium collected onto filter papers, for example.

The more sophisticated methods address the problem of the low overall efficiency of generation and acquisition of the X-ray spectrum. The low-fluorescence cross-section is addressed by using as a highly intense X-ray source, a synchrotron in the SRIXE method. The high intensity of synchrotron X-rays allows the beam to be focused and collimated whilst retaining significant intensity. The method can therefore be used in a microprobe mode, and by moving the sample in a raster pattern across the incident X-ray beam, elemental images can be generated with micron spatial-resolution. The scattered primary radiation background can be reduced by using the total-reflectance technique in TXRF [126]. The instrumental geometry limits scattering of primary X-rays in the direction of the detector, but this is at the expense of increased sample preparation. The gains in sensitivity achieved by each of these methods may soon be compounded in a method which uses a total-reflectance sample geometry in combination with a synchrotron X-ray source.

The charged-particle-beam methods EMA and PIXE also allow elemental imaging within the sample. These methods generally require that the sample be enclosed in a vacuum. The approx. 15 keV electrons used in an EMA instrument penetrate only 1–2 μm into the sample. This rapid slowing down of the charged particles generates bremsstrahlung X-rays which generate a strong background signal in the spectral region of interest. EMA thus has relatively poor detection limits. The method can be used for analysis of electro-deposits such as sources prepared for alpha-particle spectrometry where the element of interest is present at a high concentration in a very thin surface layer. The approx. 2.5 MeV-proton beam used in PIXE analysis penetrates much deeper into the sample than the EMA electron beam. The resulting proton bremsstrahlung is less intense, and backgrounds are therefore reduced. PIXE can thus achieve much lower detection limits.

PIXE [127] and SRIXE [128] have similar imaging capabilities and detection limits but both suffer from the drawback that they rely on major pieces of hardware, an accelerator in the PIXE experiment and a synchrotron X-ray source for SRIXE.

11.6.8.2 Methods based on ultraviolet or visible spectroscopy

Atomic spectroscopy in the ultraviolet and visible regions involves transitions of valence-shell electrons, and the spectra are thus sensitive to the chemical and physical form of the element of interest. For sensitive quantitative work the sample is normally

converted into free atoms in the gas phase. This can be achieved by vaporization from a furnace, by aspiration of a solution into a flame or inductively coupled plasma, by sputtering in a glow discharge, or by laser ablation. Producing free, gas-phase atoms is a particular problem for thorium, uranium, and plutonium as these elements react with traces of oxygen, even in a high-vacuum-system, to give oxides and dioxides. The following methods are based on types of atomic spectroscopy in the ultraviolet visible region:

- Atomic absorption spectroscopy (AAS)
- Graphite furnace AAS (GFAAS)
- Atomic fluorescence spectroscopy (AFS)
- Inductively-coupled-plasma optical-emission spectroscopy (ICPOES)
- Glow-discharge optical-emission spectroscopy (GDEOS)
- Laser-excited atomic-fluorescence spectroscopy (LEAFS)
- Laser-induced-breakdown spectroscopy (LIBS)
- Resonance-ionization spectroscopy (RIS)

The methods range from simple, inexpensive, absorption spectroscopy to sophisticated tuneable-laser excited fluorescence and ionization spectroscopies. AAS has been used routinely for uranium and thorium determinations (see, for example, [129]). The technique is based on measurement of the absorption of light by the sample. The incident light is normally the emission spectrum of the element of interest, generated in a hollow-cathode lamp. For isotopes having shorter half-lives than ^{238}U and ^{232}Th, this requires the construction of a hollow-cathode lamp using significant quantities of radioactive material. Measurement of technetium has been demonstrated in this way by Pollard et al. [129]. It has also been demonstrated that tuneable lasers can be used to replace hollow-cathode lamps. This avoids the safety problems involved in the construction and use of active hollow-cathode lamps. Tuneable semiconductor lasers were used by several research groups, as these are low-cost devices. They do not, however, provide complete coverage of the spectral range useful for AAS and the method has, so far, only been demonstrated for a few elements, none of which were radionuclides.

Absorption spectroscopy measures the difference in intensity between an incident-and transmitted signal. Lower detection limits can be potentially obtained by monitoring a single low-intensity signal, as in emission-or fluorescence spectroscopy. LEAFS uses tuneable lasers to efficiently excite fluorescence and, by passing the sample atoms repeatedly through excitation–fluorescence cycles, very high sensitivities can be obtained. Again, LEAFS has been demonstrated for only a limited number of elements, none of which were radionuclides. A particularly sensitive approach is to excite fluorescence by a two-photon process. In this way the wavelength of the fluorescent light is much shorter than that used to excite fluorescence, and scattered primary radiation can easily be discriminated from the fluorescence signal. As an alternative to observing the fluorescence signal in a LEAFS experiment, the state which has been resonantly-excited by a tuneable laser can be further excited by further laser photons to produce an ion. Ions can be collected and detected with electron multipliers with high efficiency leading to the extremely high sensitivity of ionization spectroscopies. LEAFS and RIS combine the high selectivity of laser spectroscopy with high sensitivities. Both these components are required to give low detection limits.

Isotope effects are observable in high resolution ultraviolet-visible spectroscopy. At

very low atomic numbers and at very high atomic numbers (especially for the actinides) isotope shifts can be observed, and coupling of the electron spin with the nuclear spin in odd-mass-number elements. Ultraviolet-visible spectroscopy can thus potentially provide isotopic information in these regions of the Periodic Table, although routine methods are not yet available. For those elements where isotopes cannot be distinguished from their simple atomic spectra an isotope-specific resonance ionization method has been suggested by Lethokov [130]. An instrument is being developed, based on this suggestion, for the determination of ^{90}Sr, in which strontium ions are accelerated to an energy of about 50 keV and neutralized collisionally. At this kinetic energy, the different strontium isotopes are travelling at sufficiently different velocities that collinear resonance ionization spectroscopy can differentiate between isotopes on the basis of their different Doppler shifts [131]. Thus, some methods of atomic spectroscopy can provide isotopic information [132].

11.6.8.2.1   Inductively   coupled   plasma–optical   emission   spectrometry   (ICP–OES)   Atomic spectroscopy is used widely in inorganic chemistry to determine total element concentrations in many sample types, and generally allows rapid sample throughput. The optical techniques permit the measurement of atomic concentrations down to sub-ng/ml levels (10^{-8} M and below) in samples of a few millilitres or less. The recent introduction of a new mass spectrometric technique allows isotope-specific measurements to be made with the ease of use and sample-throughput of the atomic spectroscopic techniques.

The inductively coupled plasma ICP is a stable argon plasma heated by inductive coupling of argon cations and free electrons, but is perhaps best though of as simply a hot flame. Temperature measurement indicates that the plasma has a temperature approaching 6000–7000 K.

Samples in solution are nebulized (at about 0.4 ml min^{-1} solution consumption) to produce an aerosol of fine droplets. A spray chamber is used to select only the smallest droplets for analysis, in practice, those below approximately 5 μm. The selected droplets are swept into the centre of the plasma by an argon stream. In the plasma, droplets undergo rapid heating causing firstly desolvation of droplets, and then breakage of molecular bonds. The resulting free atoms are electronically excited: many are ionized. As atoms leave the plasma and cool, they relax, leading to emission of light. Detection of this light is the basis of ICP–OES. The wavelengths emitted are characteristic of the elements present, and the intensity proportional to their concentrations.

ICP–OES limits of detection for many elements lie in the range 1–100 ng/ml (ppb) in solution. A few elements, notably Li, Be, Mg, Ca, Sc, Ti, Mn, Cu, Sr, Y and Ba, have limits less than 1 ng/ml. ICP–MS, on the other hand, is generally more sensitive (by 1–3 orders of magnitude) and gives isotope specific information; ICP–OES, to a first approximation only gives total element concentrations. The principal advantages of the technique are that it is multi-element and that data acquisition takes approximately 1 min with a changeover time between samples of a similar order. The technique has drawbacks: spectral interference is possible, depending on other elements present; therefore in ICP–OES a high resolution optical spectrometer may be required. The technique is best suited to solution, although direct solid-sampling techniques are being developed.

11.6.8.2.2 Laser-excited resonance ionization spectroscopy (LERIS)   To achieve both high isotopic selectivity and high sensitivity at the same time, collinear laser spectroscopy is combined with resonance ionization. The principle of resonance ionization spectroscopy is that: the atoms are excited by one or several resonant optical excitation steps into an energetically high-lying state. Subsequently, the atoms are ionized either by absorption of another photon, by collisions, or by field ionization. The photo-ions produced in this process can then be detected with high efficiency. This technique has proved to be extremely useful and sensitive in numerous applications. For the combination of collinear fast beam laser spectroscopy with resonance ionization detection, the excitation into high-lying Rydberg states, with subsequent field ionization, is best suited because of the effective suppression of background. This technique has already been applied successfully for trace-analysis of ^{3}He in environmental samples as well as for the sensitive study of radioactive Yb-isotopes at the on-line mass separator facility ISOLDE at CERN.

Monz et al. [131] have described the use of LERIS for low level detection of ^{90}Sr and ^{89}Sr in environmental samples. The details of their experimental set up are as follows: After chemical separation from the environmental sample, the Sr is inserted into the ion source. The ions are accelerated to an energy of 30 keV and pass through the mass separator, where the stable isotopes are strongly suppressed. The ^{89}Sr or ^{90}Sr ions enter the apparatus for resonance ionization in collinear geometry and are deflected by 10° to enable collinear superimposition of the laser beam. Neutralization takes place inside a charge-exchange cell filled with caesium vapour. The remaining ions are removed afterwards from the resulting fast atomic beam by various electrostatic deflectors. Subsequently, the selective excitation into high-lying Rydberg states is induced by the laser light. The Rydberg atoms are field-ionized in a longitudinal electric field and the resulting ions are deflected out of the atomic beam for counting with a particle-detector. A total isotope selectivity of $S^{88/90} > 10^{10}$ and an overall efficiency of 5×10^{-6} have been achieved. With these values, a detection limit of 1×10^8 atoms of ^{90}Sr in the presence of more than 10^{17} atoms of stable isotopes has been demonstrated. The trace-determination of such a contamination can be carried out with an accuracy of 30% within one working day, including all chemical extraction steps.

The chemical procedure for the separation of strontium from air filters is carried out without the usual addition of strontium carrier, to keep the content of stable strontium low. Such a chemical procedure has been worked out. Water and soil samples may have higher contents of stable strontium and thus require still higher values for the selectivity of the method The performance of the technique might be increased by an optimization of the ion source efficiency and higher optical excitation probability affecting both the overall efficiency and the selectivity. These improvements should enable a lowering of the detection limit for ^{90}Sr and to extend the measurements to ^{89}Sr (see also [130,133]).

11.6.8.3 Methods based on mass spectrometry

The electric and magnetic fields, used for the analysis of ions, provide only information about the two quantities E/q and M/q, where E, M and q are the energy, mass, and charge, respectively, of the ion. There are four ways in which the quantities E/q and M/q may be determined:

- magnetic selection, $(B\rho)^2 = 2(M/q)(E/q)$;
- electrostatic selection, $E\rho = 2(E/q)$;
- Cyclotron selection, $1/f = (\pi/B)(M/q)$;
- velocity selection, $v^2 = 2(E/q)(M/q)$.

where B is the magnetic field, ρ the radius of the ion path, E the electric field, f the cyclotron frequency, and v the ion velocity. Low-resolution measurements that separate neighbouring isotopes as their final output have the quantities M/q and E/q since M can be regarded as an integer, ambiguities can arise if M and q have common factors. It is for this reason that some flexibility in the choice of q is desirable. If it is possible to measure the energy of the ion also, then it is possible to determine q, and so determine the mass from the ratio M/q.

The use of energy, mass and charge signatures, at energies such that charge state 3 + or higher is dominant, is the basis for the accelerator mass spectrometry of almost all stable isotopes.

The methods listed below are based on mass spectrometry, differing mainly in the design of the ion source used:

- Spark-source mass spectrometry (SSMS)
- Glow-discharge mass spectrometry (GDMS)
- Inductively-coupled-plasma mass spectrometry (ICPMS)
- Electro-thermal vaporization–ICPMS (ETV–ICPMS)
- Thermal-ionization mass spectrometry (TIMS)
- Accelerator mass spectrometry (AMS)
- Secondary-ion mass spectrometry (SIMS)
- Secondary neutral mass spectrometry (SNMS)
- Laser mass spectrometry (LMS)
- Resonance-ionization mass spectrometry (RIMS)
- Sputter-initiated resonance-ionization spectroscopy (SIRIS)
- Laser-ablation resonance-ionization spectroscopy (LARIS)

#Let us discuss briefly the limitations of radioactive decay measurement. The observation of the radioactive decay of a single atom is possible consequently, with efficient apparatus for the detection of the decay particles and a radioactive species with a half-life of seconds and minutes, it is possible to detect all, or nearly all, of a small number of radioactive atoms in the presence of a large number of non-radioactive atoms, with radiation detection techniques. However, as the half-life increases the time taken to carry out an experiment with a small number of radioactive atoms naturally increases. For half-lives of, say, 10^6 years, efficient detection of the radioactive decay products becomes impossible unless the measurement can be continued for 10^6 years. Therefore, studies of long-lived radioactivity invariably use very large numbers of atoms, and the apparatus detects the decay of only a small fraction of the total during the experiment. In this situation, the mass spectrometric detection sensitivity surpasses by far the sensitivity of radioactive counting methods.

An important example is the study of the ^{14}C (half-life = 5730 years), generated in the atmosphere by cosmic rays, in connection of radiocarbon dating. The observed beta-ray counting rate from 1 g of contemporary carbon of biological origin is about 15 min^{-1} g^{-1}.

However, this low counting rate is supported by the presence of 6.5×10^{10} atoms of ^{14}C in the 1-g sample. If ^{14}C atoms could be counted efficiently by accelerator mass spectrometry, it would be possible to determine the ^{14}C content of very small quantities of carbon. This has now been accomplished for milligram carbon samples even, though the ratio ^{14}C/^{12}C is near or below 10^{-12}.

A comparison of measurements of long-lived radioisotopes at natural levels with beta-ray counting and AMS [134] is shown in Table 11.13.

Atomic mass spectrometry is inherently sensitive and by its nature provides isotopic information. The goal of methods of elemental analysis based on mass spectrometry is to produce a spectrum of singly charged atomic species. Again, this can be a problem for elements such as uranium, which readily form oxides. If molecular- or multiple-charged species 'contaminate' the atomic mass spectrum, they can give rise to background signals at the mass of interest or/if these molecular ions contain the element of interest, then the signal owing to that element is distributed between the atomic singly-charged ion, the multiple-charged ions, and any molecular species formed. Atomic mass spectra are simple to interpret, but great care must be taken to avoid molecular interferences especially at very low concentrations.

SIMS analysis of electrodeposited ^{232}Th alpha-particle sources gives rise to higher signals for the ThO^{+} and ThO$_2^{+}$ than for Th^{+}. This leads to difficulties in quantitation as the oxide- to atomic-ion ratios will be sensitive to local oxygen concentrations. Isobaric atomic interferences also present a problem: ^{99}Ru and ^{99}Tc, for example, have the same nominal mass and cannot be discriminated on the basis of mass except by high-resolution mass spectrometers. Even with high mass resolution, if the interfering isobar is present in excess then discrimination at high mass resolution will be difficult, and in any spectrometric method there is a trade-off between resolution and sensitivity. If there is a vast excess of an isotope of adjacent mass, even this may interfere with the signal of interest. The ability for a mass spectrometer to discriminate against such an interference is termed the 'abundance sensitivity'. Methods such as ICPMS and TIMS must discriminate against isobaric interferences by chemical separation methods prior to instrumental analysis.

TABLE 11.13

A COMPARISON OF MEASUREMENTS OF LONG-LIVED RADIOISOTOPES AT NATURAL LEVELS WITH BETA-RAY COUNTING AND AMS [134]

	Radioisotope				
	^{10}B	^{14}C	^{26}Al	^{36}Cl	^{129}I
Half-life (years)	1.6×10^6	5730	7.05×10^5	4.05×10^5	1.57×10^7
Stable isotopes	^{9}Be	^{13}C, ^{12}C	^{27}Al	^{35}Cl, ^{37}Cl	^{127}I
Stable isobar	^{10}B	^{14}N	^{26}Mg	^{36}Ar, ^{36}S	^{129}Xe
Chemical form	BeO	C	Al$_2$O$_3$	AgCl	AgI
Sample size (mg)	0.2	0.25	3	2	2
Atom per sample	2×10^5	2×10^5	4×10^5	5×10^5	2×10^6
AMS run time (min)	10	7	40	30	20
Decay counting interval (years)	110	3	250	86	1130

AMS, which is most commonly used for radiocarbon dating, discriminates against interferences in a number of ways. The ^{14}N interference in ^{14}C measurement is removed by generating a beam of anions and relying on the instability of the nitrogen anion. Molecular interferences are removed by high-energy (several MeV) collisions in a gas cell or thin foil. Further discrimination can be achieved by charge-stripping in the same collisional processes to produce highly charged ions or even ions in their maximum charge state. The combination of discrimination processes used depends upon the isotope of interest and the potential interferences. RIMS approaches the same problem by selectively ionizing only the element of interest prior to mass spectrometric separation. A selectivity of approximately 1 in 10^5 can be achieved per resonant excitation step in the ionization process and two or three such steps are frequently used. In combination with the selectivity of the mass spectrometer the method should potentially offer elemental selectivities in excess of 10^{15}.

11.6.8.3.1 ICP–MS   ICP–MS uses an inductively coupled plasma as an ion source for a mass spectrometer. The basic units of an ICP–MS system, in the order used, are the sample-introduction device, the plasma, the plasma/mass spectrometer interface, the ion focusing/ion filtering system, the detector, and the data acquisition/data handling system. Beauchemin [135] gives a helpful comparison between ICP–MS and ICP–OES.

The sample-introduction device introduces liquid samples as either a dry vapour or a fine mist into the plasma, with several options available. These include: pneumatic nebulization (the most common), ultrasonic nebulization, electrothermal vaporization (ETV, which uses a graphite furnace), flow injection [136] and direct injection [137]. The transport efficiencies of sample into the plasma for pneumatic and ultrasonic nebulization, and ETV are about 1% and 100%, respectively. Laser ablation is a common method for introducing solid samples into the plasma. This and other sampling methods for solids are reviewed by Baumann [138].

An inductively coupled argon plasma is used most frequently in ICP–MS, with argon as the cooling, carrier, and auxiliary gas. The high-temperature plasma (5000–8000 K) is sustained by radio frequency fields at the tip of a quartz torch. The plasma desolvates (if necessary), atomizes, and ionizes the sample. Horlick [139] notes the use of helium or nitrogen-based microwave-induced plasmas to eliminate interference from argon-based background species. Smith et al. [140] and Lam and McLaren [141] have used mixed carrier gases to reduce argon-based background ions.

The plasma/mass spectrometer interface allows the import of a stream of ions from the plasma, at atmospheric pressure, into the mass spectrometer, which is under vacuum. The interface has a sample cone and a skimmer cone, usually of nickel and typically with 1.0-mm and 0.8-mm apertures, respectively. The turbomolecular pump is now the most widely used type in the vacuum system.

The extraction lens, held at a negative voltage, attracts the positive ions as they emerge from the skimmer cone. The negative ions are repelled and the neutral species diffuse away. The accelerated positive ions are focused by the ion-lens stack and then enter the quadrupole region. Positioned between the extraction lens and the first ion lens, the photon stop prevents stray photons from reaching the detector. Voltages applied to the quadrupole – four metal rods mounted in a square array – produce an electric field that affects the trajectories. for any specific applied voltage, only ions of a very narrow range of mass/

charge (m/z) ratios have stable trajectories and reach the detector. A single m/z ratio may be continuously focused onto the detector, or a selection of m/z ratios can be focused sequentially onto the detector.

A channel electron multiplier, the commonly used detector, responds to each incoming positive ion and produces a measurable pulse. The number of pulses measured is proportional to the number of ions of the selected m/z ratio reaching the detector. In the pulse counting mode, maximum gain is obtained by applying a high voltage to the multiplier so that individual ion arrivals at the detector are recorded and ultimate detection limits are obtained. The analogue mode uses lower voltages, which produce lower gains. The use of lower gains extends the useful analytical range but results in higher detection limits. A Faraday cup replaces the analogue mode in some instruments.

The data acquisition/data handling system consists of a multichannel scalar (MCS) and a computer system. Signal pulses from the detector accumulate into memory channels of the MCS according to their m/z ratios. Signal pulses from replicate scans are sorted into the appropriate channels and accumulated until the analysis of that sample is complete. A computer program retrieves the totals from the MCS memory and stores the data for later manipulation or display.

As mass spectrometry has continued to gain in sensitivity and reliability, inductively coupled plasma/mass spectrometry (ICP–MS) has become increasingly useful in the measurement of radionuclides. The optimization of ICP–MS is improving our ability to use the atomic detection of radionuclides in that it allows the near-complete isotopic analysis of any form of sample. Aqueous samples are generally introduced into the plasma source, and solids or individual particles, and organic solutions, may be atomized and continuously introduced into the plasma source.

ICP–MS sensitivity, which is currently ca. 8×10^9 atoms, can be improved by:

- the use of more efficient sample introduction techniques;
- understanding of the basic principles of ion- and gas dynamics in the ICP–MS interface;
- the use of high-resolution mass spectrometers with high ion transmission.

The ultimate sensitivity could approach ca. 10^7 atoms, which would result in a superior detection capability for all radionuclides with half lives greater than 1 year.

For radionuclides with half-lives of thousands of years and longer, ICP–MS has two principal advantages over radiation counting, which are its speed of measurement and sensitivity.

Most radiation counting times range from 50 to 2500 min for most samples and most backgrounds. In contrast, an ICP–MS analysis requires only a few minutes per sample or blank, whether it is introduced via nebulizer, ETV unit, or other device. The analysis time is independent of the half-life or decay scheme of the radionuclide. The analysis time is also not greatly lengthened by a lower required MDA. Indeed, this advantage of ICP–MS over radiation counting becomes greater with increasing half-life and reducing MDA. Analysis by ICP–MS may be the preferred method, even when its sensitivity is not as great as that given by radiation counting, because of its speed.

The idea of using ICP–MS quantitatively becomes feasible for radionuclides with half-lives greater than about 1×10^3 years. However, the sensitivity that is routinely achievable with ICP–MS is not as high as that achieved with radiation counting for radio-nuclides with half-lives less than about 1×10^4 years, unless the decay scheme is unfavourable for

radiation counting. For present-day instruments, at least 10^7–10^8 atoms are necessary to quantify a nuclide by an ICP–MS with an ETV unit [142]: to use an ultrasonic nebulizer requires at least 10^8–10^9 atoms; while a pneumatic nebulizer requires at least 10^9–10^{10} atoms. To corresponding masses vary according to the atomic weight of the nuclide. (For ^{239}Pu, 2×10^7 atoms equals 8 fg.) Under favourable operating conditions, an instrument with an ETV unit should just meet an 8 fg detection limit for ^{239}Pu ($t_{1/2} = 2.41 \times 104$ years). This equals an MDA of 0.001 dpm (1.7×10^{-5} Bq), which is five times lower than the MDA given earlier. This corresponding MDA for 2×10^7 atoms of ^{240}Pu ($t_{1/2} = 6.57 \times 10^3$ years) is 0.004 dpm (6.7×10^{-5} Bq).

The sensitivity of ICP–MS for heavier elements is better than for lighter ones, owing to the lower background in the higher mass region and more stable trajectories for more massive ions [143]. This is an advantage when one is interested in analysing for long-lived radionuclides of the rare earth and heavier elements.

Essentially all of the inert sample matrix needs to be removed when performing radiation counting of all alpha-particle-emitters and low-energy beta-particle emitters because of sample self-absorption. Complete matrix removal may not be necessary for analyses by ICP–MS, depending on the elemental composition of the sample, the analytes, the sampling device, and the required sensitivity. Partial or less complex matrix decompositions and separations of analytes may suffice. For example, Hursthouse et al. [144] compare the extent of chemical purification necessary to obtain good results for ^{237}Np via ICP–MS, alpha-particle spectrometry, and neutron activation analysis. Depending on the dissolved solids content, natural waters may need only filtration and/or treatment with acid. A chemical separation of a group of elements may be satisfactory.

The preparation of purified samples for alpha-particle spectrometry is usually achieved by either electrodeposition or micro-coprecipitation. Either technique takes at least an hour. Many beta-particle emitters are precipitated with several milligrams of carrier and weighed for determining the chemical yield prior to counting, which also takes time. In contrast, a few millilitres of solution are satisfactory for ICP–MS. If the analyte concentration should exceed the linear part of the calibration curve, a simple dilution overcomes the problem.

Mixtures of beta-particle-emitting nuclides of more than one element usually have overlapping spectra. This is also often true for alpha-particle-emitting nuclides. Some of these mixtures can be analysed by ICP–MS without internal interferences. For example, the alpha-particle spectra of ^{237}Np and ^{242}Pu overlap partially even under the best of conditions, whereas ICP–MS is appropriate for analysing the long-lived radionuclides in a Np–Pu mixture. Only X- and gamma-ray counting are comparable to ICP–MS in the number of radionuclides that can be measured simultaneously.

As with all analytical techniques, ICP–MS has its problem areas; for further information see Olesik [145]. Although the mass spectrum of a sample is usually much simpler than an atomic emission spectrum for the same sample, spectral interferences from isobaric interferences and peak overlap can still be problems. Isobaric interferences result from two situations. The first occurs when two elements in a sample have nearly identical *m/z* values. An example of this is the presence of ^{113}In$^+$ interfering with the analysis of ^{113}Cd$^+$. The second situation results from the formation of 'background species'. These are ions, usually polyatomic, formed from the plasma gas alone or in combination with elements from the solvent used in the sample preparation. Examples, together with the ions

for which they cause the greatest interference, are Ar_2^+ ($^{80}Se^+$), ArO^+ ($^{56}Fe^+$), Ar^+ ($^{40}Ca^+$ and $^{40}K^+$), and O_2^+ ($^{32}S^+$). Most of these interfering species have $m/z < 81$. Fortunately, most long-lived radionuclides have masses greater than 81 amu.

Peak-overlap occurs when the major constituents in the sample have massive peaks at particular m/z values channels. Examples of this type of interference would be a massive peak for uranium at mass 238 that tails into the 237 and 239 mass channels, thus complicating the analysis of $^{237}Np^+$ or $^{239}Pu^+$. Other problems may be caused by the matrix of the sample itself. If chloride is present, a series of polyatomic chlorine-containing species may cause major interferences. As an example, $^{40}Ar^{35}Cl^+$ is an intense peak that interferes with $^{75}As^+$. Arsenic is monoisotopic, and therefore appreciable levels of chloride in the sample will seriously compromise the precise determination of arsenic. Components of the sample matrix may also contribute to oxide formation. Oxides of the form MO^+ give rise to peaks at the (m/z) + 16 position. One or more of these may interfere with nuclides of interest. An example of this is $^{48}Ti^{16}O^+$–interfering with the analysis of $^{64}Un^+$. The four other naturally occurring titanium isotopes would, of course, also give interference at their respective (m/z) + 16 values to any analytes with these masses. Formation of the oxide of the analyte also reduces the signal measured at m/z.

The sample matrix may also induce changes in the analyte signal's intensity. High concentrations of concomitant elements generally cause suppression of an analyte signal, although under certain conditions signal enhancement has been observed. In general, the lower mass elements are more subject to suppression than are higher mass elements, and higher mass elements are more likely to cause signal suppression of lower mass elements than the reverse [143].

An ICP–MS instrument will not tolerate dissolved solids at concentrations that can be run with an ICP-atomic emission spectrometer. In addition to increasing the probability of inter-element (isobaric) interferences and signal suppression, high levels of dissolved solids condense on the sample-cone orifice. This deposition degrades the sensitivity and stability of the analytical signal. Typically, a maximum of 0.1% dissolved solids is recommended for continuous nebulization with a pneumatic nebulizer. Dissolved solids should be kept below about 0.01% with an ultrasonic nebulizer, owing to its desolvation effect. Liquid samples containing up to about 1% dissolved solids can be run with ETV and flow injection. The sensitivity achievable for an element is inversely related to its ionization potential (IP). Thus, for example, the ICP–MS sensitivity for iodine (IP = 10.34 eV) is not as good as it is for nearby caesium (IP = 3.89 eV). Finally, the sample aliquot is consumed during an ICP–MS measurement, whereas with radiation-counting the sample aliquot can usually be retained and remeasured.

Some of the reported ISP-MS applications to radionuclide determination include the following.

Kim et al. [146] measured the $^{240}Pu/^{239}Pu$ ratio in two soils and an estuary silt after performing radiochemistry. They also measured this ratio in these soils by the fission track-etch technique and found that ICP–MS gave better precision. They measured the ^{239}Pu concentration on separate aliquots by alpha spectrometry, with 3.76×10^5 year ^{242}Pu tracer as the chemical yield monitor. However, at the ^{239}Pu concentrations in their samples, ICP–MS could have measured these directly.

The determination of low levels of ^{99}Tc in environmental samples (salt marsh soil, seaweed, and sea-water) by ICP–MS was reported by Nicholson et al. [147]. They employed

[95m]Tc as a yield tracer, and radiochemically isolated Tc from the matrix. They confirmed the chemical removal of any interfering isobaric [99]Ru by monitoring for other stable Ru isotopes. The chemical yield was measured by gamma-ray spectrometry. Beals [148] used 2.6-million year [97]Tc as the yield monitor in ICP–MS measurements of ^{99}Tc in river water, thereby eliminating the need for a separate yield measurement. In purified water, we have reliably detected 0.05 ng/ml of very low specific-activity [113]Cd by ICP–MS with pneumatic nebulization. For environmental waters, we believe better sensitivities could be achieved by sample concentration and a clean-up that includes the removal of interfering [113]In. It is entirely impractical to detect 0.05 ng of [113]Cd by beta-particle counting.

James et al. [149] demonstrated, with diluted aqueous standards, an MDA of approximately 8 fg of [239]Pu (1.7×10^{-5} Bq) with ETV–ICP–MS in the peak dwelling mode. Comparison measurements by ICP–MS and alpha spectrometry on radiochemically processed urine with moderately higher activity gave good agreement, considering the very low amount present. Plutonium-244 ($t_{1/2} = 8.3 \times 10^{7}$ years) is also amenable to analysis by ICP–MS, whereas 87–8 y [238]Pu is not.

ICP–MS was found to be compatible with LC for trace-metal speciation. The role of ICP–MS in trace-element speciation studies at the FSL was described [150]. The characteristics of LC–ICP–MS for the study of metalloprotein species were assessed and the chromatographic efficiency of ICP–MS was found to be similar to that obtained with a UV detector [151]. Information about the chemical nature of trace elements from food can be obtained by first treating the foods in vitro with enzymes to broadly simulate the action of enzymes in the gastrointestinal tract [152]. The soluble components can be separated by size-exclusion chromatography (SEC) and an estimate of their molecular size obtained. By coupling SEC directly to ICP–MS, the trace element content of the chromatographic fractions can also be measured. This approach has been used at the FSL to investigate the speciation of cadmium in raw and cooked pig kidney [153]. The sensitivity of ICP–MS enabled the researchers to study retail samples in which the levels of multi-element data obtained indicated that, while the feeds were contaminated with a number of elements, only lead presented a serious problem in parts of the rest of the food chain. for example, while offal samples from affected cattle were not allowed to enter the food chain, experiments with meat on contaminated bones showed that lead did not migrate significantly from the bone under a variety of cooking conditions [154].

Kim et al. [155] have reported the measurement of some long-lived radionuclides, such as ^{99}Tc, [226]Ra, [232]Th, [237]Np, [238]U, [239]Pu and [240]Pu using high-resolution inductively coupled plasma mass spectrometry (HR-ICP–MS). By using HR-ICP–MS with an ultrasonic nebulizer, the detection limits of these nuclides were 0.002–0.02 pg ml^{-1} and the sensitivities were ten times better than those obtained using HR-ICP–MS without the ultrasonic nebulizer. More accurate isotopic data were also obtained using HR-ICP–MS than with quadrupole ICP–MS at lower concentrations of the analyte because of improvement in counting statistics that can be obtained with HR-ICP–MS owing to the greater efficiency of ion transmission. A comparison of the measurement of the [240]Pu to [239]Pu ratio is shown.

Morita et al. [156] have applied inductively coupled plasma mass spectrometry (ICP–MS) to the determination of technetium-99 (^{99}Tc) in environmental samples. The determination of eliminating the interfering element (Ru) before the ICP–MS measurements are made. Technetium-95m is used as the chemical recovery tracer. Compared with conven-

tional methods, the method sensitivity is 10–100 times higher, and the counting time is 300–10 000 times shorter.

Tye and Mennie [157] have reported the development of a performance a new interface for the Plasma Quad ICP–MS, which enhances the signal-to-noise performance of the standard instrument; new high sensitivity instrument is called the Plasma Quad PQS. In order to maintain the flexibility of the instrument, the new interface is designed such that the enhanced performance can be easily switched on and off, offering the benefit of routine performance plus the high sensitivity mode when required.

The above improvements in signal-to-noise make possible the routine monitoring of many actinide elements directly by ICP–MS, potentially shortening the analytical cycle from days to hours. A further improvement on these impressive limits of detection is possible if a high efficiency nebuliser is used to introduce samples into the new instrument, giving the capability of single figure ppq detection limits in an analysis which takes minutes, not hours.

11.6.8.3.2 AMS For isotopes with long lifetimes, >1 year, it may often be advantageous to use atom-counting techniques rather than traditional decay-counting methods. This is especially true for measurements where efficiency is a criterion, as for small samples, or if high precision is required. While atom counting has a counting rate that is essentially independent of decay lifetime and sample size, the decay-counting rates are comparable only if the isotopic half-life is less than one year for a sample size of the order of 1 mg. Of course, if sufficient material is available, the decay-counting rate can always be improved by using more material [158–160].

Accelerator mass spectrometry (AMS) extends the capabilities of atom-counting using conventional mass spectrometry, by removing whole-mass molecular interferences without the need for a mass-resolution very much better than the mass-difference between the atom and its molecular isobar. This technique has been used with great success for the routine measurement of ^{14}C, ^{10}Be, ^{26}Al, ^{36}Cl and, recently, ^{129}I (see Table 11.14). Analysis of ^{14}C by AMS can, for example, generate dates with a precision that is at least equal to the best conventional beta particle counting facility. In many cases, where small sample analysis is required, the AMS method has proved superior [161]. A complete description of AMS can be found in review articles [134,159] or recent conference publications. The application of AMS to ^{129}I measurement has been discussed in detail in [160].

Accelerator mass spectrometry is an analytical technique that uses an ion accelerator and its beam transport system as an ultrasensitive mass spectrometer.

Accelerator mass spectrometry was introduced by Muller [161], who suggested that a cyclotron could be used for detecting ^{14}C, ^{10}Be and other long-lived radioisotopes, and independently by the Rochester group, which demonstrated that ^{14}C could be separated from the isobar ^{14}N by relying on the instability of the negative ion ^{14}N^{-}. Presently AMS measurements are being made at about 30 accelerator laboratories around the world, and half of these are dedicated to AMS measurements of long-lived radioisotopes.

Six long-lived radionuclides beyond uranium exist which have half-lives greater than 100 ka (^{236}Np, ^{237}Np, ^{242}Pu, ^{244}Pu and ^{248}Cm). The first two are natural by-products of the nuclear industry. Nuclear-weapons tests will generate the plutonium and curium isotopes although attempts have been to detect pre-solar-system ^{244}Pu in ores [162] or ^{244}Pu from more recent supernova debris. The detection of these isotopes is still in the development

TABLE 11.14

LONG-LIVED COSMOGENIC ISOTOPES DETECTED WITH ACCELERATOR MASS SPECTROMETRY

Isotope	Half-life (years)	Interfering stable		AMS detection limit[a]	Range of terrestrial concentration[a]
		Isotopes	Isobars		
^{10}Be	1.5×10^6	^{9}Be	^{10}B	7×10^{-15}	10^{-8}–10^{-14}
^{14}C	5.7×10^3	^{12,13}C	^{14}N	0.3×10^{-15}	10^{-12}–10^{-16}
^{26}Al	7.2×10^5	^{27}Al	^{26}Mg	10×10^{-15}	ca. 10^{-14}
^{36}Cl	3.1×10^5	^{35,37}Cl	^{36}S, ^{36}Ar	0.2×10^{-15}	10^{-12}–10^{-17}
^{41}Ca	1.3×10^5	^{40,42}Ca	^{41}K	500×10^{-15}	10^{-15}–10^{-16}
^{129}I	15.9×10^6	^{127}I	^{129}Xe	100×10^{-15}	ca. 10^{-16}

[a] Compared to the stable isotope of the same element.

stage. Unlike the natural elements, isobaric interferences are not a major problem as all isotopes will be equally rare or non-existent because of their very short decay half-lives compared to the lifetime of the solar system.

The components of accelerator mass spectrometry include: ion source, injector, tandem accelerator, positive ion analysis an detection system. A caesium sputter ion source is used for most AMS work. This is essentially a secondary ion mass spectrometry (SIMS) instrument that has been refined to produce a high current of negative ions. Generally, solid samples are used; gas samples can give intense beams, but the problem of contamination from the previous sample ('memory') is difficult to overcome. For radioisotope studies, sample size are 1–10 mg of processed material and beam currents of 1–50 μm are typical, depending on the element and ion source model. Some sort of multiple sample changing system is used at most AMS installations. For example, the main features of the 846B model high-intensity sputter source (High Voltage Engineering Europe) include: a hemispherical ionizer giving a focused Cs beam-spot of less than 0.5 mm, an *x–y* scanning stage to limit cratering effects and a 60-sample carousel with automated remote loading for throughput work. Currents of up to μA ^{12}C$^-$ have been quoted for this source from graphite targets.

Mass-analysis of the negative-ion beam with a resolution sufficient to separate isotopes of heavy elements is needed prior to acceleration. An electrostatic analyser is used at the University of Toronto to sharpen the energy distribution of ions produced from caesium sputter ion source. A pre-acceleration of the negative ion beam to 100–400 keV is used with large tandem accelerators to ensure that the injected ion beam is focused at the central terminal where the stripper canal is located.

The name 'tandem' refers to a dual acceleration design. The negative ions are accelerated to the terminal of the accelerator, which is held at a constant positive voltage, typically in the range 2–10 MV. The electron stripper at the terminal removes several electrons while energetic negative ions pass through; positive ions are then accelerated from terminal to the end of the accelerator (ground potential). Tandetrons operate reliably below 3 MV using a solid-state power supply, and tandem Van de Graaff accelerator use a rotating belt or chain to charge the terminal up to 25 MV in some models. Tandem

accelerators have the following characteristics: (i) the ion source and detector are located at ground potential for tandetrons; (ii) they do not require a pulsed beam; (iii) the electron-stripping step needed to eliminate molecules is an integral step in the operation of the accelerator; and (iv) transmission through the accelerator and subsequent analysers can be made independent of small change in the terminal voltage.

Analysers positioned after the accelerator remove scattered particles accepted by the injector analyser, molecular fragments, and unwanted charge states. Magnetic analysers alone are not sufficient. An electrostatic analyser or velocity selector is necessary to remove particles that have different masses but would otherwise have the correct mass-energy product to pass through the magnetic analysers.

At 1 MeV/amu energies, the dE/dx and total energy measurements of are made with either gas ionization detectors or silicon surface-barrier detectors, or a combination of these. The time-of-flight detector serves as an additional positive-ion mass analysis stage. It is most useful for the heaviest (slowest) ion such as ^{129}I, and consists of two time-pickoff detectors with time-resolution of a few hundred picoseconds.

Isotope ratios are obtained by alternately selecting each stable isotope and measuring its beam current in a removable or offset Faraday cup, and then by measuring the radioisotope (rare nuclide) counting rate in the detector. Standards (samples with a known isotope ratio) are used periodically for normalization, and blanks (samples containing no detectable nuclides to be measured) are used to measure the background. Ratios are corrected for time-varying linear mass fractionation when more than one stable isotope is measured, and for non-linear fractionation, which arises from the stripping process and from stray magnetic fields in the accelerator, by comparison to the standard. The precision of ratios ranges from 1% to 10%, in the AMS measurement, depending on the value of ratios and counting time (if the background is low enough). The long-lived radioisotopes ^{10}Be, ^{14}C, ^{26}Al, ^{36}Cl, ^{41}Ca and ^{129}I can now be measured in small (mg) natural samples having isotopic abundances in the range 10^{-12}–10^{-15} and as few as 10^{5} atoms.

At elevated energies (>1 MeV/amu), ions can pass through thin films or equivalent gas with virtually no attenuation of the particle beam and little energy loss. As a result of electron-capture and loss interaction an ion passing through matter is characterized by the fraction of the total ions (F_q) in a given charge state (q) where $\Sigma F_q = 1$. The resulting charge state distribution is determined by the electron-capture and -loss cross-section of an ion in a gas or solid. An equilibrium distribution will be established, whose character depends only on the ion velocity and the target material. This equilibrium distribution is independent of the initial ionic charge or the target thickness, and the approximation is valid as long as the energy loss remains insignificant.

The passage of an ion through matter with the subsequent removal of electrons from molecules will reduce the bond strength among the constituents. Generally, after a reduction of two electrons in ionically bound molecules, no bond is possible and the molecule is broken up by Coulomb force. A sufficient number of electrons may remain to leave the charged molecule in a stable or metastable configuration. To avoid the possibility of long-lived (>1 ms) metastable molecules, at least three electrons must be removed. At present, no $3+$ molecules are known to exist.

For light ions ($Z < 20$) an energy of at least 3 MeV is needed to maximize the production of charge state $+3$ ions in a gas cell. As the mean ionic charge rises approximately linearly with energy, the higher charge states will dominate at the 8 MV accelerating

potentials. Usually one of these charge states was chosen to provide the highest conversion efficiency and no molecular interference.

Separation of isobars can be accomplished by using a number of different approaches:

(i) By chemical separation. No two isobars will have the same atomic number and rarely will they belong to the same chemical group of elements. Consequently, an initial reduction of the isobaric interference is always possible by some form of chemical processing. the degree to which this is effective depends on the specific chemical differences among the isobars and the level of isobaric contamination. Because the mass ambiguity is always formed from stable elements which have had the benefit of the last $4-5 \times 10^9$ years to achieve some level of contamination in all materials, chemistry can not usually eliminate this type of ambiguity significantly below one ppb. At this level of refinement, the interference is still at least many orders of magnitude greater in concentration than the rare long-lived radioisotopes. For some very rare isobars, specifically ^{36}S (0.017%) in the case of ^{36}Cl, careful chemical processing is the primary means of isobaric reduction.

(ii) Using negative ions. Some isobars can be eliminated by exploiting the instability of negative ions. For example, noble gas negative ions are known to be metastable or unstable, thereby removing ^{36}Ar and ^{129}Xe as a source of interference in the measurement of ^{36}Cl and ^{129}I. Others such as ^{14}N$^-$ and ^{26}Mg$^-$ do not form readily or have metastable states which decay in a time scale $\ll$ 1 ms. Since the lifetimes of these metastable states are small in comparison to the transit time ($>$1 ms) through the ion-analysis system, attenuation factors in excess of 10^6 were possible with negative ions, for the detection of ^{14}C and ^{26}Al.

In contrast to the high probability for scattering and multiple charge changes for positive ions used at low energies (keV), the scattered negative ions from the more intense isobaric beams, are greatly reduced in intensity after an interaction with the residual gas in the ion source. This can be attributed to the low binding energy of the negative ions.

(iii) By full stripping of electrons. At sufficiently high energies and for cases where the radioisotope has a higher Z than the stable isobar, separation by fully stripping with subsequent magnetic analysis can be accomplished. This method has been investigated for the systems ^{3}He^{2+}–^{3}H^{1+}, ^{26}Al^{13+}–^{26}Mg^{12+}, ^{36}Cl^{17+}–^{36}S^{16+}, ^{41}Ca^{20+}–^{41}K^{19+}, ^{53}Mn^{25+}–^{53}Cr^{24+} and ^{59}Ni^{25+}–^{59}Co^{24+}. Isobar separation by full stripping allows the simultaneous acceleration of the radioactive and stable isobar, with the latter being of sufficient intensity to provide beam feedback signals.

(iv) By energy loss measurement. When ions with energies about 1 MeV/amu pass through matter, the energy loss per unit path length (dE/dx) of an ion (Z) traversing a solid or gas with velocity (V) is governed by the Bethe–Block equation,

$$dE/dx = k(Z/V)^2$$

where k is a constant. The effectiveness of energy loss measurements reduces as the atomic number increases. The percentage difference in energy loss between ^{14}C and ^{14}N is 30% at 40 MeV in isobutane gas. This rapidly decrease to 6.8% between ^{36}Cl and ^{36}S. Beyond calcium, very high energies ($>$100 MeV) are required.

(v) By gas-filled magnet. The gas-filled magnetis a powerful isobar separation instrument developed only recently for AMS. An ion passing through a gas changes its charge frequently by electron capture and loss. If this charge changing occurs

frequently enough in a magnetic field region, the trajectory is determined by the average charge state of the ion, which depends on Z. Development of the gas-filled magnet should make possible AMS of ^{36}Cl at lower energies, and aid in the AMS detection of other heavier isotopes.

(vi) By lasers. Lasers are very powerful instruments for separating elements. Since the separation of isobars from different elements is the most difficult task in AMS, the use of lasers in connection with AMS could provide a very effective clean-up of background. The basic idea in a recent proof-of-principle experiment at the Rehovot (Israel) AMS facility was to clean a negative ion beam from unwanted isobaric background ions by selective electrons detachment. ^{32}S$^-$ ions which have an electron affinity of 2.08 eV were effectively neutralized by interaction with 2.33 eV photons from a pulsed Nd:Yag laser. The same photons did not affect ^{37}Cl ions whose electron affinity is 3.62 eV. This clearly demonstrated that a laser depletion of ^{36}S background in ^{36}Cl measurements is feasible, opening the possibility for sensitive ^{36}Cl measurement at small AMS facilities where the ion energy is too low to perform isobar separation by the techniques mentioned in (iii) and (iv) above. However, for actual applications in AMS measurements, a substantial improvement in overall efficiency of the laser depletion process is necessary.

The developments in the instrumentation include the production of dedicated machines. For example, with the introduction of the 'Attomole 2000', High Voltage Engineering Europe has made available a compact ^{14}C Isotope Ratio Mass Spectrometer (^{14}C IRMS) for biomedical applications. The system provides ^{14}C/^{12}C ratios down to 10^{-13} from sub-milligram samples , typically in a few minutes. Both solid samples (carbon) as well as CO_2 can be analysed. The Attomole 2000 combines a compact instrument package with the extreme sensitivity of large tandem accelerators that are normally found in big research centres.

The main application of the instrument could be for tracer kinetic and pharmacological measurements in biomedical studies. Essentially, ^{14}C IRMS is an alternative technique to ^{13}C Isotope Ratio Mass Spectrometry (^{13}C IRMS), which is widely accepted within the biomedical community. ^{14}C IRMS is at least 100–1000 times more sensitive than ^{13}C IRMS. This allows, for example, the kinetics of ^{14}C labelled enzymes, amino acids or carcinogens to be studied in the human body at levels that are comparable to actual environmental exposure.

However, a widespread acceptance of ^{14}C IRMS (often called Accelerators Mass Spectrometry or AMS, because an accelerator is involved) in the biomedical community is at present handicapped by the size of the existing ^{14}C IRMS systems and their need for expensive support personnel. These shortcomings of the present instrumentation could be overcome with the introduction of the Attomole 2000. The system is a compact, turn key instrument that is user-friendly. Its characteristics are reflected in the following specifications:

System footprint	2.22 × 1.25 m
Sample medium	Solid graphite or CO_2
Required sample size	Solid graphite, minimum 100 μg; CO_2 sample, 0.2 μmol CO_2 (minimum 10× modern ^{14}C content)
Output	^{14}C/^{12}C ratios
Accuracy	Better than 3%
Counting rate	>250 counts s^{-1} for 10× modern samples

Background	Less than 10^{-13} for $^{14}C/^{12}C$ ratios (ca. 0.1 modern)
Detection efficiency	1–2%
Detection limit	approx. 4 attomole (10^{18} mole) ^{14}C for 3% accuracy
Throughput	400 sample day^{-1} (based on samples with an average of 4× modern ^{14}C content)

Furthermore, the system is fully atomized, self tuning, and needs little or no maintenance. The operator will consider the instrument as an analytical tool; the fact that an accelerator is involved is incidental. Up to 50 solid graphite samples can be loaded in a carousel prior to analysis. CO_2 samples can be admitted on line to the ion source. The ion source uses a primary caesium beam to sputter the sample under investigation to form a negative carbon-ion beam. The ion beam is accelerated through the system to reach the detector with an energy of 2.5 MeV.

11.6.8.4 Laser-induced photoacoustic spectroscopy (LPAS)

Growing interest has been directed recently to the application of photoacoustic sensing techniques to the spectroscopic analysis of various optical absorbers in very dilute concentrations. For this purpose a laser is commonly used as a light source. Since the discovery of the photoacoustic effect by Bell [163], its application has a long history of development. Renewed interest in photoacoustics has emerged, starting with the work of Kreuzer [164] who analysed trace amounts of gas molecules by laser-induced photoacoustic generation. The theory, instrumentation, and application of laser-induced photoacoustic generation developed in recent years have been thoroughly reviewed by Patel and Tam [165] and more recently by Tam [166,167]. Other reviews are also available,by different authors [168–176].

Because of difficulties involved in handling radioactive preparations, the photoacoustic sensing technique had not been applied until a few years ago to the spectroscopy of aqueous actinide ions. A relatively simple detection apparatus using photoacoustic spectroscopy has been developed for spectral work on actinide ions, using a pulsed laser as a light source. This detection apparatus can be used for radioactive α-emitting aqueous samples without restriction to corrosive solutions and facilitates the spectroscopic investigation of actinide solutions, particularly transuranic ions, in very dilute concentrations. The spectroscopic system has more recently been introduced into different nuclear chemical laboratories and further developed for a variety of purposes. Most of these developments are confined primarily to the spectroscopic investigation (i.e., speciation) of actinides in very dilute solutions, or natural aquatic systems in which the solubility of actinides is, in general, very low ($<10^{-6}$ mol l^{-1}). Optical spectroscopy of high sensitivity is an indispensable tool for the study of the chemical behaviour of actinides in natural aquatic systems, which is a newly developing research field in connection with nuclear-waste disposal in the geosphere. For this reason, not only is photoacoustic spectroscopy attracting great attention, but also thermal lensing spectroscopy and fluorescence spectroscopy, all of which use laser light sources, are growing in use for the same purpose.

Actinides have particular spectroscopic properties which are characterized primarily by the $f \rightarrow f$ transitions within the partially filled $5f$ shell and thus by a number of relatively weak sharp absorption bands. The optical spectra of actinides are characteristic for their

oxidation states, and to a lesser degree dependent upon the chemical environment of the ion. Thus spectroscopic investigation provides information on the oxidation state of an actinide element and also serves to characterize the chemical states, such as hydrolysis products, various complexes and colloids. Hence, laser-induced photoacoustic spectroscopy with its high sensitivity can be used conveniently for the speciation of aqueous actinides at very dilute concentrations.

For a summary of the present knowledge of laser-induced photoacoustic spectroscopy, as regards theoretical backgrounds, instrumentation and radiochemical applications to particular problems in aquatic actinide chemistry, see the paper by Kim et al. [177]. Since there is no other radiochemical application known in the literature, apart from the measurement of tritium decay by a acoustic sensing technique, the present discussion is limited to applications to actinide chemistry, particularly in aquatic systems. The most interesting field of application is, and will be, the geochemical study of long-lived radionuclides, namely man-made elements (transuraniums). The main importance of such a study is not only the detection of a migrational quantity of radioactivity but also the characterization of chemical states, and hence their chemical behaviour in given aquifer systems. Knowledge of this kind will facilitate a better prediction of the environmental impact of transuranic elements which are being produced in ever growing quantities and will be disposed of in the geosphere.

Since LPAS application to actinide chemistry is in its infancy, only a limited number of works are available in the published literature. Experiments performed so far are confined to hydrolysis, complexation reactions with carbonate, EDTA and humate ligands, and a variety of speciation works for Am(III) – and to much lesser extent – for U(IV), U(VI); Np(IV), Np(V), Np(VI); Pu(IV) and Pu(VI). Of considerable interest is the LPAS application to the direct speciation of actinides in natural aquifer systems, where the solubility of actinides is, in general, very low and multicomponent constituent elements and compounds are in much higher concentrations than actinide solubilities. The study of the chemical behaviour of actinides in such natural environments requires a selective spectroscopic method of high sensitivity. LPAS is an invaluable method for this purpose, but its application to the problem is only beginning.

11.6.9 QA/QC procedures

Quality assurance of the determination of radionuclides in food and environmental samples ensures that the quality of data obtained is maintained at an adequate confidence level. This has to be evaluated objectively. Quality assurance includes quality control, which involves all those actions by which the adequacy of equipment, instruments, and procedures are assessed against established requirements. For the purpose of quality assurance, the following items must be ensured: that the equipment and instruments function correctly; that the procedures are correctly established and implemented; the analyses are performed correctly; errors are limited; records are correctly and promptly maintained; the required accuracy of measurements is maintained, and systematic errors do not arise.

In general, the design of a quality assurance programme should take the following factors into account:

- quality of equipment and instruments;

- training and experience of personnel;
- verification of procedures, by routine analysis of control samples and the use of standard methods for analysis;
- frequency of calibration and maintenance of equipment and instruments – the variability in the measuring system is an important aspect of this;
- the need for traceability of the results of determinations to a national standard;
- the degree of documentation needed to demonstrate that the required quality has been achieved and maintained.

It is important to have each item of the quality assurance programme established. Intercomparison is also necessary to evaluate generally the quality assurance of the determinations. By this process, it is possible for data to be compared between laboratories or within a laboratory at different times.

It is essential to develop a quality assurance (QA) programme that covers sample-collection, sample-handling, methods for on-site and laboratory analysis, data handling and record keeping. The QA programme should address the variety of different scenarios likely to be encountered. Appropriate calibration and analytical standards and a variety of reference materials will be needed. To keep costs down, one should design carefully a QA programme that recognizes that for some signatures high precision data are not required. If, for example, one analyses for a typical short-lived radionuclide which does not exist in nature, background measurements are unnecessary, however low the reported concentration. In other cases, where one looks for faint anomalies in certain isotope ratios, the QA programme should demand a knowledge of background values and their variability; this would be much more expensive.

The protocols should include 'blank' samples as well as 'background' samples. In the case that an attempt is made to find an undeclared facility adjacent to a declared one, the analyst should try to take 'background' samples from a plant somewhere else, which is similar to that part of the installation which is being examined. When attempting to find an undeclared nuclear facility at a declared site, the optimum background samples would be from similar facilities which are a part of the declared installation.

Reliability of results is a function of precision (reproducibility) and accuracy (true value). The precision of results can be determined easily by internal measurement. The determination of accuracy in most cases, however, requires more detailed procedures such as the following:

- Analysis by as many different methods, analysts, and techniques as possible. In cases where agreement is good, the results are assumed to be accurate
- Control by as many different methods, analysts and techniques as possible. In cases where agreement is good, the results are assumed to be accurate
- Control-analysis with reference materials that are as similar as possible to the materials to be analysed. Agreement between certified and observed values is then a direct measure of accuracy for that particular determination
- Participation in an interlaboratory comparison. Samples used in such an inter-comparison should be, as far as possible, similar in composition and concentration to the samples to be analysed on a routine basis. The agreement between the results received from a particular laboratory and the most probable mean value obtained from statistical

evaluations of all the results will be a measure of the accuracy for that particular determination

11.6.9.1 Reference materials

For practical reasons, most analytical laboratories are not in a position to check accuracy internally, without an external source of reference materials. To overcome some of the difficulties in checking the accuracy of analytical results, the IAEA provides the Analytical Quality Control Services (AQCS) Programme to assist laboratories in assessing the quality of their work. AQCS coordinates intercomparison studies and supplies reference materials. Participation is on a voluntary basis and at minimum cost. Information supplied by laboratories taking part in the intercomparisons is treated as confidential.

The IAEA has traditionally played an important role in the development and testing of analytical methodology for the determination of radionuclides and, through the AQCS programme, provides a service by offering laboratories the option of determining their accuracy by distributing reference and intercomparison materials containing radionuclides in different types of materials. The analytes of interest in these samples include naturally occurring radionuclides and radionuclides of fission and activation products.

The IAEA AQCS Programme provides three main types of material.

- Materials that can be used in analytical laboratories working in the fields of nuclear technology and isotope hydrology. These include uranium ore reference materials and other substances relevant to nuclear fuel technology, as well as stable isotope reference materials for mass spectrometric determination of isotope ratios in natural waters
- Materials with known contents of uranium, thorium and/or transuranic elements or fission products for the determination of environmental radioactivity or control of nuclear safety
- Materials for use in the determination of stable trace elements in environmental or biomedical research. Radiochemical methods such as neutron activation or isotope-dilution analysis, are often used in the determination of such trace elements and constitute an important contribution of nuclear techniques to applied science [178]

Table 11.15 lists the radionuclides referenced by IAEA, their activity, matrix, and sample code. Table 11.15 also includes materials of marine origin. The intercomparison samples cover a range of materials, and contain radionuclides with very different levels.

Orders for reference- and intercomparison materials are currently running at the level of about 3000 units year^{-1} for the whole AQCS programme. The distribution of reference and intercomparison materials is coordinated by the Chemistry Unit of the Agency's Laboratories at Seibersdorf, but it also receives input from other Sections of the IAEA, including the Hydrology Section, the Nutrition and Health Related Environmental Studies Section, the Safeguards Analytical Laboratory, Monaco.

An overview of the types of RMs in the non-nuclear field available for monitoring and the preparation of natural matrix reference materials as a result of intercomparisons containing radionuclides, is given in this chapter. The latter type of material gained widespread interest and importance after the Chernobyl accident. It is expected that similar

TABLE 11.15

RADIONUCLIDES REFERENCED BY IAEA

Referenced analyte	Activity or concentration	Confidence (Bq kg^{-1})	Matrix interval	Reference date	Sample code
^{40}K	391	379–405	Tuna homogenate, Mediterranean	1 January 1989	IAEA-352
	527	510–543	Milk powder	31 August 1987	IAEA-152
	150	141–161	Sea-plant, *Posidonia oceanica*	1 January 1988	A-14
	220	189–226	Sediment, marine	1 January 1985	IAEA-307
	240	211–269	Sediment, lake	31 January 1986	SD-N-2
	272	252–299	Fish flesh	1 January 1986	SL-2
	539	510–574	Milk powder	31 August 1987	MA-B-3/RN
	552	563–569	Milk powder	1 January 1990	IAEA-321
	657	637–676	Clover	1 August 1986	IAEA-156
	1381	1320–1456	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
	1575×10^3	1511–1644	Hay powder	31 August 1987	IAEA-154
^{90}Sr	1.5	1.33–1.57	Milk powder	31 August 1987	A-14
	3.3	3.16–3.44	Milk powder	1 January 1990	IAEA-321
	6.9	6.0–8.0	Hay powder	31 August 1987	IAEA-154
	7.7	7.0–8.3	Milk powder	31 August 1987	IAEA-152
	14.8	13.4–16.3	Clover	1 August 1986	IAEA-156
	30.34	24.2–31.67	Soil	30 January 1983	SOIL-6
	54.8	46.3–59.2	Bone, animal	15 December 1981	A-12
^{106}Ru	23	22–25	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
	33.5	30.0–36.5	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
^{110m}Ag	20	1.0–2.27	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
	5.1	4.8–5.5	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
^{134}Cs	1.6	1.5–1.8	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
	1.6	1.5–1.9	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
	15.5	14.8–16.2	Milk powder	1 January 1990	IAEA-321
	132	126–138	Clover	1 August 1986	IAEA-156
	764	722–802	Milk powder	31 August 1987	IAEA-152
	1355	1295–1417	Whey powder	31 August 1987	IAEA-154
^{137}Cs	2.7	2.5–2.8	Tuna homogenate, Mediterranean	1 January 1989	IAEA-352
	0.8	0.5–1.0	Sediment, marine	1 January 1985	SD-N-2
	1.79	1.62–1.97	Milk powder	31 August 1987	A-14
	2.4	2.2–2.6	Sediment, lake	31 August 1986	SL-2
	4.9	4.5–5.2	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307

TABLE 11.15 (continued)

Referenced analyte	Activity or concentration	Confidence (Bq kg^{-1})	Matrix interval	Reference date	Sample code
	5.6	5.3–6.0	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
	14.2	13.7–15.3	Fish flesh	1 January 1986	MA-B-3/RN
	53.65	51.43–57.91	Soil	30 January 1983	SOIL-6
	72.6	71.1–74.2	Milk powder	1 January 1990	IAEA-321
	264	254–274	Clover	1 August 1986	IAEA-156
	2159	2503–2209	Milk powder	31 August 1987	IAEA-152
	3749	3613–3887	Hay powder	31 August 1987	IAEA-154
^{210}Pb	0.6	0.36–1.0	Tuna homogenate, Mediterranien	1 January 1989	IAEA-352
	73	66–75	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
^{210}Po	2.2	1.7–2.7	Tuna homogenate, Mediterranean	1 January 1989	IAEA-352
^{226}Ra	3.1	2.1–4.4	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
	5.2	4.4–6.7	Bone, animal	15 December 1981	A-12
	79.92	69.56–93.43	Soil	30 January 1983	SOIL-6
	269	250–287	Soil	30 January 1988	IAEA-312
	342	307–379	Sediment, stream	30 January 1988	IAEA-313
	732	678–787	Sediment, stream	30 January 1988	IAEA-314
^{228}Th	25	2.2–3.6	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
^{232}Th[a]	4.9	4.5–5.4	Sediment, marine	1 January 1985	SD-N-2
^{238}Pu	0.017	0.016–0.023	Sea-weeds, Mediterranean	1 January 1988	IAEA-308
	0.025	0.022–0.028	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
^{239}Pu	1.04	0.962–1.11	Soil	30 January 1983	SOIL-6
^{239}Pu	8.8	6.51–9.0	Sediment, marine	1 January 1985	SD-N-2
	0.50	0.46–0.52	Sea-weeds, Mediterranean	1 January 1988	IAEA-307
	0.72	0.66–0.79	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
^{241}Am	0.036	0.030–0.050	Sea-plant, *Posidonia oceanica*	1 January 1988	IAEA-307
	0.17	0.16–0.25	Sea-weeds, Mediterranean	1 January 1988	IAEA-308

[a] The ^{232}Th is in equilibrium with ^{228}Ra and ^{228}Th.

materials with natural and elevated levels of radionuclides will be of importance for decades to come.

Reference materials for radioactivity measurements can also be obtained from the following specialized international or national organizations.

1. Central Bureau for Nuclear Measurements, Commission of the European Communities, Joint Research Centre, Geel (Belgium)
2. Office des Rayonnements Ionisants Commissariat a l'Energie Atomique BP 21, 91910, Gif-Sur Yvette (France)
3. Commission d'Etablissement des Methodes d'Analyse Commissariat a l'Energie Atomique BP 6, 92265, Fontenay aux Roses (France)
4. AEA Fuel Services, Chemistry Division, Harwell Laboratory, Oxfordshile OX11 0EA (UK)
5. New Brunswick Laboratory, U.S. Department of Energy, 9800 South Cass Avenue Argonne, IL 60439-4899 (USA).
6. All Union Foreign Economic Association 'Techsnabexport', Staromonetniy Per. 26, 109180 Moscow (USSR).

11.6.9.2 Intercomparison

Intercomparison studies organized over the last twenty years are generally based on recommendations of consultants' group meetings, working groups convened by the Director General of the IAEA, and in response to the demands of many of the IAEA Member States for assistance in developing methodologies for the measurement of radioactivity. The Chemistry Unit of the Agency's Laboratories at Seibersdorf, Austria distributes every 4 years a questionnaire concerning the need for organizing intercomparison tests and the preparation of reference materials. Using these data, the AQCS programme collects different kinds of environmental and foodstuff bulk samples, some of which were affected by fallout radioactivity following the Chernobyl nuclear reactor accident. The general policy is to organize intercomparisons with those materials which are in most demand and have various levels of activity. Collection of a sufficient quantity of the raw materials (typically of the order of 200–400 kg) is first organized. The samples obtained by a sampling operation are generally dried, ground and homogenized. Aliquots are then taken at this stage and analysed to check the homogeneity of the bulk materials. Other preparation steps include aliquoting into bottles in amounts of about 25–100 g per bottle. To ensure long-term stability of the material, the sealed bottles are sterilized by gamma-ray irradiation (^{60}Co at a dose of 2.5 megarads). A further control of homogeneity takes place after the materials have been distributed into bottles. Within-bottle and between bottle homogeneity is determined separately, usually by determining ^{40}K, ^{137}Cs, ^{90}Sr and U. When this has been done, the material is announced in the AQCS Catalogue as an intercomparison material.

Participants in such intercomparisons are provided with information about the material and special forms on which they are requested to report, for each element, up to six individual net results on a dry-weight basis, the sample-weights used, information about the analytical method, and various other items. To preserve anonymity, each participant is assigned a code number, known only to himself and the AQCS programme, by which he is identified in the report that is subsequently prepared on the results of the intercomparisons. The number of participants in each intercomparison varies, but at present is around 50.

A list of some of the materials for intercomparisons which have been organized by the AQCS programme is given in Table 11.16.

The results submitted by the participants are in all cases evaluated by the AQCS programme. A specific feature of any intercomparison is that gross errors occur quite frequently, and results differing by as much as two or three orders of magnitude may be reported by participating laboratories. Various approaches and criteria for the detection and rejection of the highest and the lowest values or outliers have been discussed in the literature [17]. The analytical data received in intercomparison exercises by the AQCS programme are treated using two different methods in order to derive a consensus value, which is considered to be a reliable estimate of the true value. The first method was suggested by Dybczynski [179]. Another method used for testing of outliers is based on non-parametric distribution. Outliers are eliminated by the application of a test based on Tchebycheff's inequality.

In applying the first method, four different criteria, namely Dixon's test, Grubbs' test, the coefficient-of skewness test and the coefficient of kurtosis test, are used at a signifi-

TABLE 11.16

SOME OF THE IAEA INTERCOMPARISON EXERCISES INVOLVING RADIONUCLIDES

Matrix	Level	IAEA code	Year	Certified as RM
Alga, marine	Environmental	AG-B-1	1983	+
Milk powder	Environmental	A-14	1983	+
Sediment, marine	Environmental	SD-N-1/2	1983	+
Soil	Environmental	Soil-6	1983	+
Sediment, marine	Environmental	SD-N-2	1983	+
Fish flesh	Environmental	MA-B-3/RM	1986	+
Sediment, deep sea	Environmental	SD-A-1	1986	+
Sediment, lake	Environmental	SL-2	1986	+
Air-filter, simulated	Artificial	IAEA-083	1986	+
Milk powder	Elevated[a]	IAEA-152	1987	+
Whey powder	Elevated[a]	IAEA-154	1987	+
Soil	Environmental	IAEA-312	1988	+
Sediment, stream	Environmental	IAEA-313	1988	+
Sediment, stream	Environmental	IAEA-314	1988	+
Milk powder	Environmental	IAEA-321	1988	+
Clover	Elevated[a]	IAEA-156	1988	+
Sea-weeds, mediterranean	Elevated[a]	IAEA-308	1988	+
Sediment, *baltic sea*	Elevated[a]	IAEA-306	1988	+
Sea plant, *posidonia oceanica*	Elevated[a]	IAEA-307	1988	+
Uranium ore, phosphate	Environmental	IAEA-364	1989	+
Tuna homogenate, mediterranean	Natural	IAEA-352	1989	+
Sediment, pacific ocean	Elevated[a]	IAEA-368	1990	+
Soil	Elevated[a]	IAEA-375	1991–92	−
Grass	Elevated[a]	IAEA-373	1991–92	−
Cockle flesh	Environmental	IAEA-134	1992	−
Sediment, marine	Environmental	IAEA-135	1992	−

[a] Contaminated with radioactive fallout from Chernobyl.

cance level of $\alpha = 0.05$. If a laboratory-mean for each element as a single unweighted value was declared to be an outlier by any criterion, it is rejected and the whole procedure repeated until no more outliers could be identified. The remaining laboratory means are then combined in the usual way to provide estimates of the overall mean (consensus value) and its associated standard deviation, standard error, and 95% confidence interval.

The consensus values cannot automatically be accepted as recommended to certified values because their analytical validity usually requires a re-assessment in the light of additional analytical information, such as concentration level, number of different analytical methods used, percent of outliers, and other criteria. In practice, certified or recommended values are always based on the following requirements. Data should be available from a certain number of participants and two or more different analytical methods; there should be no significant differences between the groups of accepted results; outliers should not exceed 20–30% of the submitted results. Depending on the extent to which the data satisfy such acceptance criteria, the consensus values are then assigned to one of the following conclusions: certified or recommended concentration, information value, or not recommended. More information about these criteria may be found in the Agency's reports published after each intercomparison; these are free of charge.

The Agency's Analytical Quality Control Services (AQCS) programme provides mainly four types of materials:

- materials which can be used in analytical laboratories working in the fields of nuclear technology and isotope hydrology: these include uranium-ore reference materials and other substances of interest for nuclear fuel technology, as well as stable isotope reference materials for mass spectrometric determination of isotope ratios in natural waters;
- materials with known content of uranium, thorium and/or transuranium elements, or fission products for the determination of environmental radioactivity or control of nuclear safety;
- materials for use in the determination of stable trace elements in environmental, biomedical and marine research.
- materials which can be used in analytical laboratories working in the fields of monitoring organic microcontaminants in the marine environment.

Many countries practice national intercomparison programmes. For example, the Japanese nation-wide intercomparison program is based on the following:

- *Comparison method.* Two methods of comparison, the 'sample dividing method' and the 'reference sample method', were adopted for comparing the results of radionuclide analysis.
- *Item for analysis and measurement method.* Gamma spectrometry is used. Participating laboratories are requested to determine artificial radionuclides as ^{40}K, ^{54}Mn, ^{59}Fe, ^{60}Co, ^{131}I, ^{137}Cs, ^{144}Ce, for the 'reference sample method', but as ^{40}K and ^{137}Cs for the 'sample dividing method'.
- *Samples and materials for intercomparison.* The environmental samples are soil, milk and crops. The reference samples are agar gel, alumina powder and liquid milk, which are all spiked with known radioisotopes.

The intercomparison exercises show a need for greater standardization of the analytical

techniques used for radionuclide determination. This is indicated in McGee et al. [180], where the bias and measurement errors in radioactivity data from four European radiation research laboratories were reported.

Within the framework of the International Chernobyl Project, the IAEA's Seibersdorf Laboratories organized an intercalibration exercise [181] among some of the laboratories which were involved in assessing the environmental contamination in the former USSR by the accident. The objective was to assess the reliability of the radioanalytical data for food and environmental samples, which were used to assess the doses. The initial study reference materials from the stocks of the IAEA's Analytical Quality Control Service (AQCS) were re-labelled and submitted to 71 laboratories as blind samples. These natural matrix materials included samples of milk (containing two different levels of radioactivity), soil, air-filters, and clover. The concentrations of radionuclides in these samples were known from previous intercalibration exercises. The overall range in performance was broad, which is as observed in previous international intercomparisons.

The Central Service for Protection against Ionizing Radiation (SCPRI), a service of the French Ministry of Public Health, National Institute of Health and Medical Research, was nominated at the End of 1969 as the International Reference Centre (IRC) of the World Health Organization, for Radioactivity measurements.

Four laboratories in the world, namely the Radiation Protection Bureau in Ottawa (Canada), National Institute of Radiation Protection in Stockholm (Sweden), Environmental Monitoring and Support Laboratory (EPA) in Las Vegas (USA), and the National Radiation Laboratory in Christchurch (New Zealand) have been officially designated as WHO collaborating laboratories. At the present time, 28 laboratories from 17 countries are interested in the WHO–IRC intercomparisons. Its program of intercomparison shows the following characteristics:

- the radioactivity of the samples is the present environmental monitoring level;
- generally, the samples present real radioactivity resulting from fallout or releases from nuclear facilities;
- the amount of the product provided allows several tests to be carried out;
- standard materials can be provided;
- a preliminary study of the results of each intercomparison is given to the participants as soon as possible.

In this programme, a wide variety of samples has been adopted since 1970. These include liquid milk, animal bones, human bones, foods, low-level radioactive liquid waste, groundwater, mineral water, river sediment, sea-weed, pond water, freshwater fish, cereals, seawater, rain-water, drinking-water, soil, and vegetation.

Concerning the general conditions of the intercomparison programmes in progress and the results obtained, it can be noted that:

- the IRC has diversified its program by introducing new categories of samples (waters from various origins, sediments, fish, sea-weed, liquid waste, cereals, and soil, etc.) in which laboratories involved in environmental monitoring of nuclear power plants are interested;
- with regard to the quality of the analyses, the situation presented in the preceding annual report has not evolved much;

• the regular participation of different laboratories in the intercomparisons provides a comprehensive view of their technical capabilities and of the quality of their analytical work.

11.7 CONCLUSIONS

Many radionuclides, both natural and man-made, are distributed through all the compartments of the environment. They contribute to the dose received by man either by inhalation, digestion or direct exposure. Therefore, it is of the utmost importance to develop experimental procedures capable of measuring even minute activities of all radio-isotopes.

In this chapter, we have shown that such capabilities exist and that the progress in this field will result in reduced minimum detection limits. An important aspect of this type of measurement is quality control, which has been receiving increased attention.

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Chapter 12

Determination of organic pollutants in industrial wastewater effluents

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CONTENTS

12.1 GENERAL INTRODUCTION

Many sites are contaminated by old landfills where industrial wastes were buried without consideration of the risk of polluting the soil and groundwaters. Thousands of landfills of urban and industrial waste have been identified in western countries. In the USA, the Environmental Protection Agency estimated the existence of over 100 000 industrial disposal sites [1]. The number and the nature of landfills pose a high environmental risk and there is much concern about the possible detrimental effects of the pollutants contained within the discharge on human health. The US law known as the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA, also called 'Superfund') was the first major response to the problem of hazardous waste [2]. Within the European Union (EU) and USA several parameters are considered when monitoring the leachate of landfills and waters subject to pollution by it, such as the electric conductivity and conduction, and the concentrations of chlorides, cyanides, fluorides, sulfates, sulfides,

TABLE 12.1

PARAMETERS USED FOR THE ANALYSIS OF A LEACHATE ACCORDING TO USEPA AND EU REGU-
LATIONS [1]

Parameter	Typical values	Range
PH	6	5.3–8.5
Conductivity (μS/cm)	4000	2500–9000
Residue 180°C (mg/l)	500	200–1000
Total hardness (mg/l $CaCO_3$)	3500	300–10000
COD	18000	3000–45000
BOD_5	10000	2000–30000
Alkalinity (mg/l $CaCO_3$)	3000	1000–10000
Organic nitrogen	200	1–600
Nitrates (mg/l NO_3)	25	5–40
Ammonia (mg/l NH_4)	200	10–800
Chlorides (mg/l)	500	10–3000
Sulfates (mg/l)	300	100–1500
Calcium (mg/l)	1000	200–3000
Iron (mg/l)	300	200–1700
Total phosphorus (mg/l)	25	1–70
Lead (mg/l)	0.5	0–2.00
Cadmium (mg/l)	0.05	0–0.05

nitrites, pesticides and solvents. Table 12.1 shows the values for several general para-
meters in leachates. However, industrial waste landfills usually contain many other
compounds and it is impossible to list them all. Polar organic pollutants in untreated
industrial wastewaters may give rise to problems due to their low bio-degradability and
toxicity. Industrial effluents might contain natural and synthetic dissolved organic
compounds with dissolved organic carbon (DOC) values varying between 100 up to
3000 mg/l. More than 95% of this organic content is due to polar, ionic and highly
water soluble compounds [3]. Therefore, application of the appropriate analytical techni-
ques to get strict characterization of contaminated effluents needs to be done in order to
gain a picture of the potential pollution of a landfill. In this respect the EU promulgated
several years ago the so called 'black list' of 132 dangerous substances (Directive 76/464/
CEE) of target analytes that should be monitored as dangerous substances discharged into
the aquatic environment [4]. Among the list there are included several organo-halogens for
example polychlorinated biphenyls (PCBs), chlorotoluenes, chloropropanes, some orga-
nophosphorus compounds like pesticides or tributylphosphate, chlorophenols and poly-
cyclic aromatic hydrocarbons (PAHs). A new Directive on Integrated Pollution Prevention
Control (IPPC) was promulgated in 1996 by the European Union [5] expanding the range
of pollutants that should be monitored in industrial effluents discharges. This Directive
involves a multi-annual work program that covers many industrial sectors like the paper
and pulp industry, refineries and textiles. It is indicated in the Directive that all the
substances discharged by the different industrial sectors should be monitored and the
former European Union black list expanded by adding new compounds. Therefore, it is
of interest to the EU to develop monitoring strategies in order to characterize wastewaters
where routine methods had failed to account for a majority of TOC. The outcome is that

the main part of pollutants in industrial discharges are not typically sought or identified. Failure to implement procedures to detect these diverse compounds may allow their release and dispersion to go unnoticed in the environment. This may result in severe health, environmental and economic consequences.

Effort in the implementation of different approaches and techniques for the analysis of organic contaminants contained in industrial discharges is being made towards solving the described problem. In this sense, new analytical procedures are being developed, based on the use of efficient preconcentration and clean-up tools together with separation and determination techniques with a high capability of identification of organic pollutants.

Regarding the isolation of organic compounds from wastewaters, solid phase extraction (SPE)-based methods are gaining acceptance as an alternative to traditional liquid-liquid extraction (LLE) protocols. SPE allows automatization of the extraction process overcoming the drawbacks related to LLE such as manipulation of large volumes of generally toxic and inflammable organic solvents and extensive labour, time and glassware [6]. Advanced SPE methods, based on the coupling of different sorbents, allow one to obtain fractionated extracts containing different contaminants ready to be analysed by the most suitable analytical technique [7,8].

Following SPE, the most widely used separation methods are liquid chromatography (LC) or gas chromatography (GC) coupled to mass spectometry (MS) for the determination of target and non target analytes. A variety of non- to medium polar compounds in wastes from hazardous dump sites have been determined by routine and well established GC–MS techniques. For example, Reemtsma et al. [9] used GC–MS in the electron impact (EI) mode with derivatization to trimethylsilyl derivatives for the characterization of tannery wastewaters whereas groundwater samples from Superfund sites were characterized by means of GC–MS using a special capillary column designed for polar organic compounds [3]. GC–MS with chemical ionization was used by Betowski et al. for the analysis of hazardous waste samples [10]. Recently, the development of LC–MS interfaces has led to rugged and reliable LC–MS-based techniques enabling the determination of polar, ionic, heavy and thermally unstable compounds in industrial discharges. Characterization of nonionic and anionic surfactants and polar related compounds using atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) was reported [11].

Nevertheless, the complex chemical nature of industrial emissions is a limitation to chemical analyses in their ability to totally characterize the chemical composition of these mixtures and leads to the need of a subsequent chemical-specific genotoxicity or carcinogenicity assessment. In contrast to chemical analyses, bioassays provide a means of assessing complex mixture toxicity without a prior knowledge about the chemical composition of the mixture [12]. Advanced methodologies integrate chemical and toxicity studies for global characterization of the organic content and potential health effects of industrial effluents [13,14]. For example, bioassay-directed chemical fractionation permits the isolation and identification of defined chemical fractions that contain genotoxic activity [15]. Other biological techniques have appeared as complementary tools to chemical analysis for characterization of wastewaters. This is the case for enzyme-linked immunosorbent assay (ELISA) tests and biosensor devices. The high increase in the use of ELISA tests is likely due to the ease and cost-effectiveness of this type of assay. The lack of sensitivity of the some common GC or LC detectors in complex matrices has led to a

growing interest in developing new simple, inexpensive and sensitive detection principles such as sensors based on biological recognition reaction as enzyme-based biosensors [16,17].

In summary, this chapter focuses on the description of the methodology for the analysis of target and non target compounds present in wastewaters such as untreated or treated industrial effluents and leachates from landfills. In the sections following, sample preparation strategy, separation and identification methods, and non conventional analytical techniques, such as toxicity-based or biological methods, are successively discussed. Applications of the techniques developed for the analysis of wastewaters are included.

12.2 SAMPLE PREPARATION METHODS

Determination of target compounds in wastewaters is possible after separation from liquid accompanying matrices and its interferences. Therefore, separation of the organic components from water accomplishes two purposes: (i) it removes interfering substances; and (ii) at the same time concentrates target analytes in order to make analysis possible. It is not surprising that considerable effort has been put into methods of separation and concentration as wastewater matrices are too complex for any analytical method that requires clean extracts. In this section the most important preconcentration techniques are discussed.

Traditional methods for isolation of hazardous organic compounds from wastewater are variations of the acid/base/neutral liquid-liquid extraction (LLE) [18]. This procedure involves large volumes of generally toxic and inflammable organic solvents and extensive labour, time and glassware. In addition, each step in the sample preparation may introduce contamination or increase sample loss. Alternative solid-phase extraction (SPE) methods overcome these problems and allow to get effective extraction of target analytes and sample clean-up in a single step. A wide range of sorbent materials is commercially available in different configurations and with appropriate particle size. Due to all the presented advantages, SPE is becoming the method of choice for isolation of organic components from aqueous samples. The use of alkyl-bonded silica is recommended for the preconcentration of medium-polarity analytes whereas more hydrophobic polymeric sorbents based on styrene-divinylbenzene (SDB) resins are the most commonly employed material for polar compounds. Graphitized carbon black is also efficient for the extraction of polar analytes although it may present problems of irreversible adsorptions for some compounds and therefore elution should be performed in backflush mode. Different ion exchange materials are available for the extraction of ionic industrial pollutants. For example, octadecyl-bonded silica (C18) has proved efficient for the extraction of organic pollutants in wastewater if a maximum load of 7 mg DOC per 100 mg of SPE material was applied [19]. Nevertheless, this sorbent material (C18) had low extraction efficiency for the more highly water-soluble phenols which were successively preconcentrated in a graphitized carbon black (GCB) reversible cartridge [20]. These polar phenolic compounds were also extracted by means of polymeric sorbents [21] based on SDB resins leading to good recovery values as shown in Table 12.2 where a comparison of extraction efficiency of different sorbent materials is presented. Linear alkylbenzenesulfonates (LAS)

TABLE 12.2

MEAN RECOVERIES OBTAINED FOR THE PRECONCENTRATION OF SOME POLAR PHENOLIC COMPOUNDS USING A POLYMERIC SORBENT [21], GRAPHITIZED CARBON BLACK [20] AND OCTADECYLSILICA [19][a]

Compound	Sorbent		
	SDB polymer (Lichrolut EN)	GCB	C18
Phenol	79	76	3
4-Nitrophenol	89	101	8
2-Chlorophenol	84	99	11
2,4-Dinitrophenol	79	98	15
2-Nitrophenol	80	98	13
2,4-Dimethylphenol	86	100	29
4-Chloro-3-methylphenol	86	99	56
2,4-Dichlorophenol	80	100	65
2,4,6-Trichlorophenol	100	98	95
Pentachlorophenol	89	97	95

[a] Spiking level: 5 ppb. Loading volume: 1 l for SDB and 2 l for GCB and C18.

were concentrated in a SAX strong anionic exchanger with average recoveries of 97% for the most common commercial LAS [22].

A method for the preconcentration of a variety of common organic contaminants among them phenolic compounds, benzophenone, isothiocyanate–cyclohexane, ethylbenzoate, benzidines, acridine, 1,1,3,3-tetramethyl-2-thiourea, 2,2-dimethyl-1,3-propanediol, phosphates and phthalates was developed [23]. Target compounds were chosen according to the origin of the samples and considering a compendium of contaminants commonly found in chemical disposal sites [1]. For the SPE process, a SDB-based cartridge (Lichrolut EN) was conditioned with 7 ml of methanol and 3 ml of water at 1 ml/min. Different volumes (300, 500 and 900 ml) of water sample spiked at 50 μg/l with target analytes were loaded at 15 ml/min in the polymeric cartridges which were eluted with 2 × 5 ml of acetonitrile. The final evaporation of the extra solvent to a final volume of 2 ml was carried out with a stream of nitrogen. Table 12.3 shows the main recoveries and relative standard deviation (RSD%) obtained for the target compounds in the preconcentration of different sample volumes. Recoveries varying from 70% to 104% were obtained for the preconcentration of 900 ml for the most non-polar compounds (e.g. benzidines) although the most polar compounds such as catechol and 2,2-dimethyl-1,3-propanediol were not detected due to breakthrough. Recoveries varying between 22% and 55% were obtained for these compounds in the preconcentration of 300 ml of wastewater sample. Consequently, the results indicated that reliable detection of all compounds was only feasible by loading volumes of 300 ml at most. Therefore, loading volumes lower than 300 ml were recommended in order to retain a maximum number of pollutants as the main problem related to industrial waste water samples is not low concentration of contaminants but their incomplete identification mainly due to losses in the analytical process. The matrix effect on the efficiency of the SPE process was noted: in general lower recoveries than those

TABLE 12.3

MEAN RECOVERIES AND RSD% ($n = 3$) OBTAINED IN LOADING DIFFERENT VOLUMES OF WATER SPIKED AT 50 μg/l WITH THE TARGET ANALYTES USING OFF-LINE SPE WITH A SDB-BASED POLYMERIC SORBENT [23]

Compound	V_{load} 300 ml	V_{load} 500 ml	V_{load} 900 ml
Catechol	55 (13)	<5	<5
Phenol	57 (17)	<5	<5
Acridine	55 (15)	<5	<5
4-Methylphenol	63 (11)	24 (11)	<5
2,4-Dinitrophenol	59 (11)	32 (15)	23 (13)
2,2′-Biphenol	88 (10)	66 (11)	43 (12)
4-Nitrophenol	79 (13)	64 (12)	48 (13)
3,3′-Dichlorobenzidine	108 (9)	104 (9)	51 (12)
2-Nitrophenol	89 (11)	97 (12)	71 (13)
Naphthol	97 (8)	107 (9)	69 (9)
Benzidine	86 (9)	95 (8)	77 (9)
1-Methylindol	92 (8)	97 (9)	95 (10)
Benzophenone	105 (11)	104 (9)	103 (10)
3,3′-Dimethylbenzidine	103 (9)	99 (10)	104 (10)
Dibutylphthalate	54 (7)	48 (10)	41 (9)
Dimethylphthalate	69 (15)	55 (13)	46 (9)
4-Nonylphenol	34 (5)	26 (6)	13 (9)
Pentachlorophenol	87 (9)	85 (8)	79 (11)
2,2-Dimethyl-1,3-propanediol	24 (9)	11 (9)	<5
Bis(2-ethylhexyl)phthalate	63 (9)	58 (10)	44 (9)

obtained in other water matrices were obtained. This is due to the fact that wastewater is characterized by the presence of interferences and particles that can decrease the effectiveness of the SPE process by plugging the pores of the sorbent and diminishing the active surface area of the sorbent and therefore accelerating breakthrough of target analytes.

Advanced SPE methodologies involved fractionation of the extracts according to its polarity and/or toxicity. Very complex samples with fractionation requirements were extracted by means of acid/base/neutral LLE in order to eliminate interferences [24] and reduce the complexity of the fractions of interest [25]. As an alternative to this laborious LLE process, a sequential SPE (SSPE) approach based on the use of different sorbents in series and differential elution was developed. Medium-polarity organic compounds were preconcentrated in an octadecylsilica sorbent bed and desorbed separately using solvents of different polarity (from hexane to methanol). The most polar organic compounds in the acidified water residue were trapped by a polymeric sorbent [7]. This method has the advantage of allowing to get analyte group fractionation and leading to improved recovery values as compared to those obtained by single SPE. Fig. 12.1 summarizes the recoveries obtained for target analytes in each fraction and Fig. 12.2 shows a flow-chart of the SSPE procedure including sorbent phases, their elution and the compound classes found in the respective fraction. The comparison between the SSPE procedure and the simple SPE with polymeric sorbents in terms of recovery is illustrated in Table 12.4. Higher recoveries were obtained in all cases by means of the SSPE method and
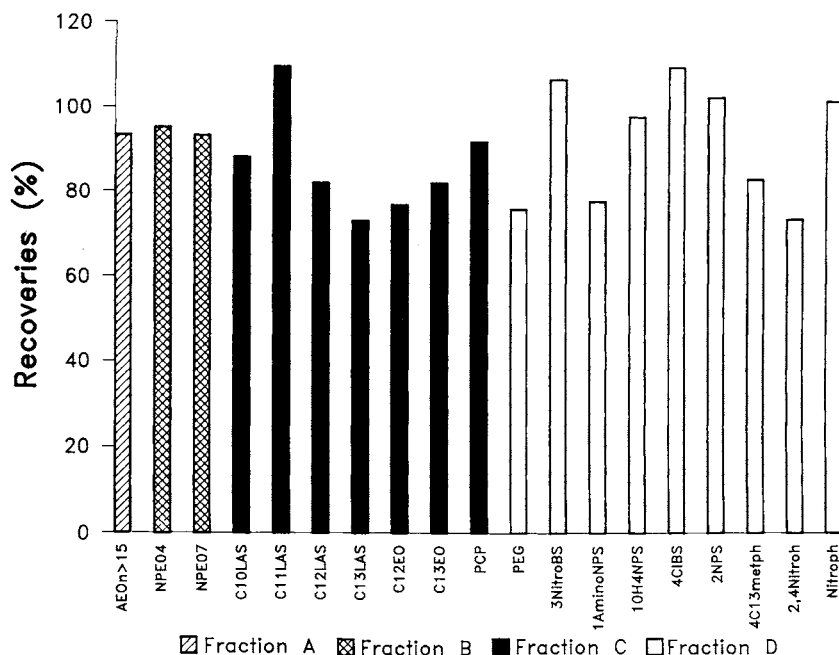
Fig. 12.1. Main recoveries obtained for different classes of compounds in each fraction of the SSPE protocol.

additionally it allowed to detect more compounds and to efficiently eliminate interferences. Other SPE-based fractionation methodologies integrate toxicity studies in the extraction process. This is the basis of bioassay-directed chemical analysis, a protocol used to identify specific chemicals or classes of chemicals contributing most to the toxicity of a complex mixture. The method involves SPE of organic material followed by a series of fractionation steps in which toxicity of the different fractions is measured and the most toxic ones are retained for further analysis [26]. In this sense, various methods have been developed in order to identify different types of toxicants. For example, a toxicity-based method to identify non polar organic contaminants in effluents using cladocerans and fishes as test organisms and GC–MS to identify the toxicants was developed [13]. This method used SPE with C18 cartridges for preconcentration of toxicants that were desorbed by passing a graded sequence of methanol/water solvents through the column. Toxicity testing was performed on the whole effluent, the filtered effluent, the post-SPE effluent and the eight methanol/water fractions. Toxic fractions were further split by HPLC fractionation and measured for toxicity. To illustrate the method, toxicity data are presented in Table 12.5 for the SPE fractionation of an effluent containing nonpolar toxicants. In this effluent sample, toxicity was observed in the 75%, 80%, 85% and 90% methanol/water fractions which were combined and then fractionated. GC–MS analyses of the final extracts revealed the presence of carbaryl, diazinon and chlorfenvinphos whose concentrations were responsible for the effluent toxicity. In another approach [14], a screening protocol that employs SPE for separating liquid wastes into fractions which are assessed for toxicity using *Daphnia magnia* immobilization test was presented. In the first stage a
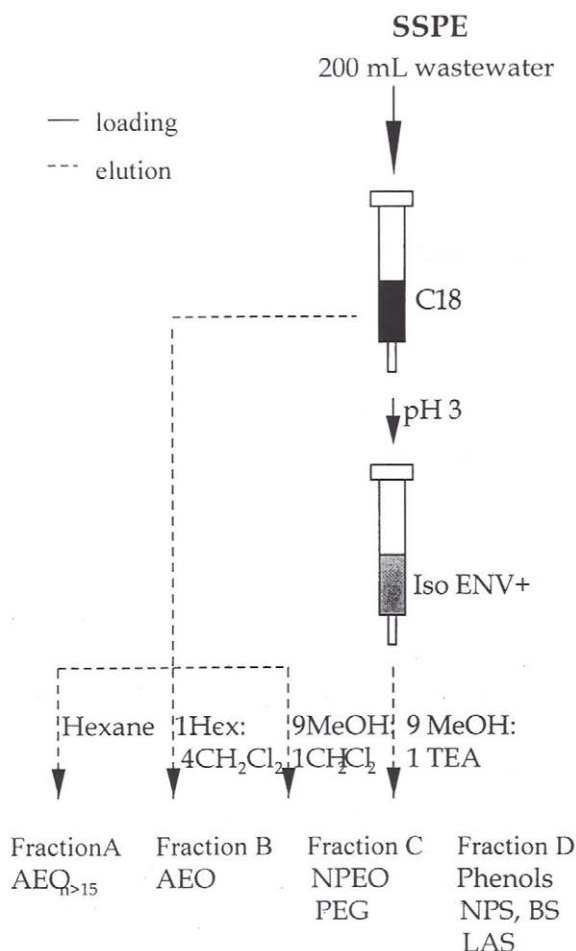
Fig. 12.2. Flow-chart of the SSPE procedure.

SPE with polymeric sorbents was applied to separate highly polar organic and inorganic species (pass) from less polar organic chemicals (extract). Preparative HPLC then separated the extract into fractions of increasing hydrophobicity (K_{ow}). The pass, containing the most water-soluble compounds, was then tested after EDTA addition to reduce toxicity and thereby asses the contribution of heavy metals to toxicity. The fraction with highest K_{ow} range was the most toxic to *Daphnia* and GC–MS was employed to characterize its toxic components. Pass was analysed by atomic spectrometry showing that copper can explain about 3% of the measured toxicity while the contribution of the other trace metals is almost negligible. LC–MS analysis were performed on the most toxic polar extracts. An scheme of the proposed protocol is illustrated in Fig. 12.3.

Although many sample pretreatments are done with off-line disposable columns, the use of on-line techniques is favoured because of the total automation of the process. An on-line precolumn fitted in a LC injector was used for preconcentration of wastewater samples in

TABLE 12.4

COMPARISON OF THE PERFORMANCE OF A SINGLE POLYMERIC CARTRIDGE SPE METHOD AND A SSPE METHOD APPLIED TO THE CHARACTERIZATION OF THE SAME TANNERY WASTEWATER; RESPONSE OBTAINED FOR THOSE COMPOUNDS EXTRACTED WITH BOTH TECHNIQUES [7][a]

Retrieved compound	Response single SPE	Response sequential SPE
Polyethoxylated (MOE 4) glycol (PEG$_4$)	+ + +	+ + + + +
Cyclohexane carboxylic acid	+ + + + +	+ + + + +
2-methylthiobenzothiazole (MTBT)	+ + +	+ + + + +
Nitrophenol	+ + + +	+ + + + +
Nonylphenol polyethoxylate NPE$_5$ (MOE 5)	+	+ + + + +
Dodecylic alcohol polyethoxylate AL$_{12, 4}$ (MOE 4)	+	+ + + + +
Tridecylic alcohol polyethoxylate AL$_{13, 4}$ (MOE 4)	+	+ + + + +
Tetradecylic alcohol polyethoxylate AL$_{14, 4}$ (MOE 4)	+	+ + + + +
Diethylhexylphthalate (DEHP)	+ +	+ + + + +

[a] +, very low (<10%); + +, low (10–50%); + + +, medium (50–70%); + + + +, high (70–90%); + + + + +, very high (>90%).

loading position and thereafter specific compounds were desorbed by the mobile phase in injection position [27]. Unattended analysis was possible and easily done by using a time-based valve and solvent switching program. Combination of different types of precolumns, optimized for pollutants of special interest, gave satisfactory group separations prior to analysis. In this sense, the sample was pumped through the series of precolumns in the order C18-PRP$_1$ (a SDB-based resin)–Aminex A5 (a resin-based sulfonic acid cationic exchanger). The first C18 precolumn trapped the nonpolar dye stuffs; the second PRP$_1$

TABLE 12.5

TOXICITY TESTS PERFORMED ON AN INDUSTRIAL EFFLUENT AND ITS C18 FRACTIONS WITH *Ceriodaphnia dubia* WITH 48 h DURATION [13][a]

Sample	Toxicity[a] (% LC$_{50}$)
Whole effluent	35
Filtered effluent	39
Post C18 SPE effluent	NM
C18 SPE fractions (%methanol)	
25	NM
50	NM
75	23
80	35
85	27
90	71
95	NM
100	NM

[a] NM, no mortality. Control measurements gave no mortality.

Fig. 12.3. Flow-chart of the toxicity-based procedure for detecting toxic components in wastewaters.

precolumn trapped the more polar aromatics like *p*-chloro- and *p*-nitrophenol and *p*-chloroaniline; and the third cationic exchanger precolumn retained polar anilines [28].

The complexity of wastewater samples demands high specificity of the sample pretreatment process and as a consequence other nonconventional extraction techniques have been developed. This is the case of membrane extraction that is especially useful when on-line monitoring is required such as for example in the optimization of industrial processes and minimization of the emissions. The basis of this technique is the selective transport of analyte molecules across a semipermeable membrane. Those compounds which permeate the membrane from the sample are removed at the other side of the membrane with an extracting liquid. The driving force for the extraction is related to the diffusion coefficient and the ratio of the partition coefficient of the analyte between the sample matrix and the membrane on one side of the membrane and between the membrane and the extractant on the other side. This device is especially suitable for wastewater as it provides an excellent interface between 'dirty' wastewater samples and the analyser since it excludes particulates, dissolved inorganic compounds and many other interents. A membrane/liquid chromatographic analyser was developed for use on-line at a wastewater treatment plant for the determination of chlorinated phenols at low μg/l levels in the plant effluents [29].

Solid phase microextraction (SPME) with polyacrylated-coated fibres is gaining acceptance due to their advantages as a fast, solvent-free, portable and inexpensive sampling technique. In SPME, the organic pollutants are adsorbed from the matrix by the solid-phase coating from which they are thermally desorbed and transferred to a GC injector. This technique has been successively applied to the analysis of polychlorinated butadienes [30] and aromatic amines [31] in wastewaters by means of polydimethylsiloxane, polyacrylate, polydimethylsiloxane/divinylbenzene, carbowax/divinylbenzene and carbon

fibres. A new interface has been developed to couple SPME sampling technique with narrow-bore high-performance liquid chromatography. This interface maintains the advantages of SPME and in addition, it provides possibilities for the analysis of semi- and nonvolatile organic compounds in wastewater [32].

12.3 MASS SPECTROMETRIC CHARACTERIZATION: GC–MS AND LC–MS

Analysis of organic pollutants in industrial effluents requires procedures that are both flexible and capable of detecting specific pollutants. Therefore, a substance-specific detector is required with all the separation techniques; otherwise a large number of standards is indispensable. In this sense, mass spectrometry (MS) is the analytical solution for the identification of target and non target pollutants in industrial discharges. The technique can be combined on-line with the most important chromatographic separation techniques applied in wastewater samples: gas chromatography (GC) and liquid chromatography (LC).

Gas chromatography–mass spectrometry (GC–MS) is the most widely used technique for characterization of wastewaters. It provides useful spectroscopic information that can be compared with libraries that now include more than 200 000 spectra.

Most common GC–MS techniques involve electron impact (EI) ionization where ion formation is a function of the energy of ionizing electrons in the electron impact process. In this case, the electrons are provided by a heated filament in the evacuated ion source and accelerated through a potential and directed across the chamber where they may hit the analyte molecules. This technique was applied to the determination of organic components in leachates from hazardous waste disposal sites revealing the major presence of several organic phosphates and some phthalates. Most alcohols, phenols, carbonyl compounds and lower fatty acids coming from the natural decay of organic matter were also detected [33]. The number of polar substances detected was more than of non-polar species and although no two landfills are the same, this is a general trend for industrial wastewater. Frequently, chromatograms and mass spectra of these hazardous waste extracts contain interferences that make qualitative assignments doubtful and quantifications difficult. Alternative ionization techniques can aid in the detection, confirmation and quantification of compounds in environmental and hazardous waste samples.

Chemical ionization (CI) mass spectrometry has been used extensively as a supplementary method to electron impact (EI) mass spectrometry. Using a protonating agent at relative high pressure in an ion source promotes ion-molecule reactions which can result in the formation of abundant protonated molecules of the compound of interest. Since proton transfer under CI mass spectrometric conditions is a 'softer' process than EI mass spectrometry in terms of the average amount of energy deposited in a molecule, these ions are expected to produce less fragmentation resulting in more molecular weight information. Combination of CI with quadrupole mass spectrometry technology enables dual acquisition of both positive and negative ion data aiding in the confirmation of compounds detected by EI GC–MS [10]. In general, by GC–MS a variety of non and medium polar compounds are generally determined in waste waters and effluents like phthalates, organic phosphates, benzenes, polyaromatic hydrocarbons (PAHs) [1,25,34]. This fact is illustrated in Table 12.6 where some of the compounds identified in industrial discharges

TABLE 12.6

LISTING OF INDIVIDUAL OR CLASSES OF POLLUTANTS REPEATEDLY IDENTIFIED BY GC–MS-BASED METHODS IN INDUSTRIAL WASTEWATERS AND THEIR RANGE OF CONTENTS

Compound	Content[a]	Reference
Short-chain carboxylic acids (C5 to C8)	+ + + +	[8,33,34]
Fatty acids (C9 to C18)	+ + +	[8,34]
Aliphatic dicarboxylic acids (C5 to C11)	+ + +	[8]
Cyclohexanes		
3,5,5-Trimethylcyclohexanone	+	[8]
3,5,5-Trimethylcyclohex-2-en-1-one	+ + + +	[8]
3,5,5-Trimethylcyclohexanol	+ + +	[8]
Cyclohexanecarboxylic acid	+ +	[8]
Aromatic carboxylic acids		[25]
Benzoic acid	+ + + +	[8]
Phenylacetic acid	+ + + +	[8,34]
Phenylpropionic acid	+ + + +	[8]
Methoxycinnamic acid	+ +	[8]
Phenols		
Phenol	+ + + +	[1,8,33]
p-Cresol	+ + + +	[8,33]
p-Chloro-m-cresol	+ +	[8,25,34]
Alkylphenol	+ + +	[1,25]
p-tert-Butylphenol	+ +	[33]
Chlorinated phenols	+ + +	[33,34]
Naphthol	+ +	[33]
Brominated phenols	+ +	[33]
Nitrophenols	+ + +	[33]
Bisphenol A derivatives	+ +	[1,8,33]
Alcohols		
Hexanol	+ + +	[8]
2-Ethylhexanol	+ + + +	[8]
1,6-Hexandiol	+ + +	[8]
Glycerol	+ +	[8]
Indoles	+ + +	[1,8,25]
Aliphatic hydroxylated carboxylic acids (C5 to C8)	+ + +	[8]
Aromatic hydroxylated carboxylic acids (C5 to C8)	+ + +	[8]
Phthalates		[8,34]
Dialkylphthalates	+ + +	[33]
Di(2-ethylhexyl)phthalate	+ + + +	[33]
Polyaromatic hydrocarbons (PAHs)	+ +	[25,33,34]
Aliphatic hydrocarbons (C10 to C32)	+ +	[33,34]

TABLE 12.6 (*continued*)

Compound	Content[a]	Reference
Chlorinated isocyanates	+	[25]
Aromatic ketones	+ + +	[25]
Aliphatic ketones	+ + +	[1]
Chlorinated anilines	+ +	[25,33]
Thiophenes	+ +	[25,33,34]
Alkylbenzenes	+ +	[25]
Alkylnaphthalenes	+ + +	[1,25,33]
Phosphates		
Tris(2-butoxyethyl)phosphate	+ + + +	[8,33]
Tris(1,3-dichloro-2-propyl)phosphate	+ +	[33]
Tris(2-chloroethyl)phosphate	+ +	[33]
Xylenols	+ +	[25,33]
1,4-Dioxane	+	[33]
Benzothiazole derivatives	+ +	[1,8,33,34]
Caffeine	+ +	[33]
Chlorinated benzenes	+ + +	[1,33]

[a] (+) µg/l; (++) 10 µg/l; (+++) 100 µg/l; (++++) 1 mg/l.

by GC–MS are listed. This table includes commonly occurring contaminants but it should be taken into account that origin specific pollutants not included in this list could be present depending on the type of industry discharge. Therefore, a great number of compounds may be detected by GC–MS; however many polar, ionic, heavy and thermally unstable compounds cannot be analysed by these techniques. A different approach should be used for these pollutants which usually comprise more than 95% of the organic content [3]. Specially designed GC columns have been used for the analysis of major polar compounds in wastewaters, for example carboxylic acids, alcohols and ketones were detected by GC–MS using a nitroterephthalic acid-modified polyethylene glycol phase column with improved resolution and peak shape as compared to the results obtained with standard columns [3]. In another approach, more than 50 compounds including *n*-alkanes, phthalates, esters, acids and phenols were identified in industrial effluents by means of high-temperature GC–MS with column phases that can be operated at temperatures up to 370–420°C allowing the analysis of high molecular weight compounds [35]. In spite of the effort put on the analysis of polar compounds from industrial dumpings by GC–MS, the most suitable techniques are those based on liquid chromatography (LC) coupled to mass spectrometry.

LC techniques have several advantages over GC: less sample clean up is required, thermally labile compounds are more easily analysed, derivatization is usually not required and polar and high molecular weight compounds can be identified. LC–MS has not been so commonly used for the characterization of polar analytes in industrial effluents as GC–MS.

Prior LC–MS applications involved the used of particle beam (PB) or thermospray (TS) interfaces. In a TS interface, a jet of vapour and small droplets is formed out of a heated vaporizer tube. Nebulization takes place as a result of the disruption of the liquid by the expanding vapour that is formed upon evaporation of part of the liquid in the tube. The ionization of the analytes is due to chemical ionization reactions and ion evaporation processes involving the use of a volatile buffer dissolved in the eluant as CI reagent. In a PB interface, nebulization is obtained either pneumatically or by TS nebulization, into a near atmospheric pressure desolvation chamber which is connected to a momentum separator where the high molecular-weight analytes are preferentially transferred to the MS ion source, while the low-molecular-weight solvent molecules are efficiently pumped away. The analyte molecules are transferred in small particles to a conventional EI/CI ion source. In this respect, the United States Environmental Protection Agency (USEPA) has published two methods for the analysis of solid waste (SW-846), involving either particle beam (method 8325) [36] or thermospray (method 8321) [37]. These methods involve the determination of different kinds of pollutants including disperse azo dyes, phosphates, pesticides and benzidines. Both methods were recently applied to the characterization of many organic acids found in Superfund sites [3]. LC–TS–MS was used to confirm that approximately half of the unidentified total organic halocarbon (TOX) content in leachates from a hazardous waste site is 4-chlorobenzene sulfonic acid [38]. LC–PB–MS has been used for the determination of low volatility (isocyanate derivatives), thermally unstable (phenylurea herbicides) and highly polar (phenoxyacetic acid herbicides) compounds. The method took advantage of the existence of library searchable electron impact mass spectra for a range of samples in various matrices [39]. However, the lack of structural information or sensitivity related to TS and PB, respectively, and the poor repeatability and reproducibility of the obtained results was the main obstacle to routine analytical applications of LC–MS in prior works.

Nowadays, the development of atmospheric pressure ionization (API) LC–MS interfaces has led to rugged and reliable LC–MS interfaces. API LC–MS interfaces provide structural information similar to that obtained by chemical ionization techniques and overcome the limitations of previous LC–MS interfacing devices. Electrospray ionization (ESI) is based on the application of a high electric potential to a solvent emerging from a capillary causing the solvent to break into fine threads which disintegrate into small droplets due to autorepulsion of the electrostatically charged surface that overcome the cohesive forces of surface tension. An important limitation of this technique (ES) is the need of a low flow rate (0.01 ml/min for conventional ES and 0.05–0.06 ml/min for pneumatically assisted electrospray–ESI) forcing the use of a postcolumn split device, therefore affecting sensitivity and reproducibility of the analytical method. Under ESI–MS, 90% of the observed ions are due to ions present in solution, therefore this technique permit to detect specifically those compounds that produce permanently charged species in solution [40]. As compared to ESI, atmospheric pressure chemical ionization (APCI) offers in general, more structural information due to the additional heat supply in the nebulization process and it is more a gas phase chemical process. Therefore APCI interface has more general application than ESI although it does not provide ESI's sensitivity. In spite of the great advances in API interfaces, few studies using LC–MS have yet been performed for the analysis of wastewater. In most cases, LC–ESI–MS was used such as for the determination of nonionic polyethoxylated surfactants [41] and its degradation

products polyethylene glycols and related acidic forms [42]. Another group of anionic pollutants, alkyl ethoxysulfates and its metabolites alkylsulfates were determined by LC–ESI–MS. This technique resulted an appropriate method for trace-level quantification of such charged, nonvolatile compounds as it permits to detect these compounds that produce permanently charged species in solution [43]. In a recent work, LC–ESI–MS was applied to the determination of linear alkylbenzene sulfonates (LAS), a group of widespread surfactants used for household as well as industrial applications [44]. A variation of this method was needed for the determination of highly polar naphthalene- and benzene-sulfonates. In this case, ion-pair chromatography was employed for the separation of target compounds and a post-column device for increasing the organic content of the mobile phase in order to improve the sensitivity of the ESI–MS detector was used [45]. As compared to ESI, APCI is not so specific for charged compounds in solution but in general, it offers the possibility of determining a wider range of organic pollutants. LC–APCI–MS has been used for the determination of nitro- and chlorophenols present in pulp effluents at levels ranging from 4 to 27 μg/l [21]. It was a good alternative for the identification of nitrophenols as its derivatization for the GC–MS detection is not straightforward. Poly-ethoxylated nonionic surfactants and related compounds were determined in very complex tannery wastewater by means of LC-APCI-MS [7]. Preliminary flow injection analyses (FIA) for detecting polyethoxylate nonionic surfactants were performed in positive-ioni-zation mode at 30 V for the injection of 20 μl of 10 mg/l methanolic solution of the most common industrially used polyethoxylated nonionic surfactants. Table 12.7 reports the fragmentation pattern followed by target analytes in the FIA experiment. In all cases, the homologous series for each surfactant approximates a statistically normal distribution. Spectra show clearly the $[M + H]^+$ ion for each compound with equidistant signals at 44 m/z units relative to the various ethoxylate oligomers. Na^+ adducts appear together with these peaks and differing 22 m/z values from them. Apart from these peaks common for all the studied surfactants, other series of peaks have been detected and in summary, char-acteristic peaks for polyethoxylated nonionic surfactants are those at 133 and 177 m/z units. In order to distinguish between the different functionalities, the more abundant peaks typical for each type of studied surfactant should be monitored. For example, the presence of peaks at m/z 151 and 195 should be checked for alcohol polyethoxylates $(AEO_{n,x})$; at m/z 271 and 291 for nonylphenol polyethoxylates $(NPEO_x)$ and at m/z 101 and 145 for polyethylene glycols (PEG_x).This technique enabled to obtain both molecular weight and structural information by raising the cone voltage to control the extent of fragmentation [7]. An LC–APCI–MS-based protocol permitted unequivocal identification of polar analytes such as isothiocyanate-cyclohexane, ethylbenzoate, 2-methylbenzene-sulfonamide, tetramethyl-thiourea, 2,2-dimethyl-1,3-propanediol and 1-methyl-2-pyrroli-dinone and other medium polar compounds among them pentachlorophenol, tributyl phosphate, 4-nonylphenol and some phthalates at concentration levels varying from 0.16 to 54.5 μg/l [23]. Two ionization modes (positive-ion and negative-ion) and different cone voltage (20 and 40 V) were tested leading to the results shown in Table 12.8. In general, more fragmentation was obtained via collision-induced dissociation (CID) by raising the cone voltage which allows to get structural information for the identification of unknowns. However, an extraction voltage of 20 V was preferred because it led to higher sensitivity than 40 V with enough structural information in both NI and PI modes. It was interesting to note that $[M + H]^+$ or $[M - H]^-$ ions were detected in PI and NI modes,

TABLE 12.7

LC–APCI–MS SPECTRA OF POLYETHOXYLATED NON IONIC SURFACTANTS (10 mg/l) IN PI MODE WITH CONE VOLTAGE SET AT 30 V USING AS ELUANTS 50% H_2O AND 50% MEOH:ACN (1:1) BOTH ACIDIFIED WITH 0.5% OF ACETIC ACID [7]

Compound	Characteristic m/z ions	m/z ions for n, x
Aliphatic alcohol polyethoxylate	$\overset{H}{\underset{H}{>}} \overset{+}{O} (CH_2CH_2O)_x H$	$n = 10$: Decylic alcohol $141 = {}^+C_{10}H_{21}$
	107	$C_{10}H_{21} O (CH_2CH_2O)_x CH_2\overset{+}{C}H_2$
$C_nH_{2n+1}(OCH_2CH_2)_xOH$ AEO$_{n, x}$	151 (107 + 44) 195 (151 + 44) 239 (195 + 44) 283 (239 + 44) 327 (283 + 44)	185 ($x = 0$) 229 ($x = 1$) 273 ($x = 2$) $x = 3$ 291 ± 44 $[M + H]^+ \pm 44$ $x = 6$ 423 ± 44 $[M + H]^+ \pm 44$ $x = 10$ 598 ± 44 $[M + H]^+ \pm 44$ $n = 12$: Lauric alcohol
	$HO (CH_2CH_2O)_x CH_2\overset{+}{C}H_2$	
	89 ($x = 1$) 133 ($x = 2$)	$169 = {}^+C_{12}H_{25}$ $C_{12}H_{25} O (CH_2CH_2O)_x CH_2\overset{+}{C}H_2$
	177 ($x = 3$) 221 ($x = 4$)	213 ($x = 0$) 257 ($x = 1$) $x = 4$ 363 ± 44 $[M + H]^+ \pm 44$ $n = 13$: Isotridecylic alcohol

$C_{13}H_{27} O (CH_2CH_2O)_x CH_2\overset{+}{C}H_2$

271 ($x = 0$)
$x = 5$ 421 ± 44 $[M + H]^+ \pm 44$
$x = 9$ 597 ± 44 $[M + H]^+ \pm 44$
$x = 4$ 397 ± 44 $[M + H]^+ \pm 44$
$x = 4$ 419 ± 44 $[M + Na]^+ \pm 44$
$x = 6$ 485 ± 44 $[M + H]^+ \pm 44$

Nonylphenol polyethoxylate

$127 = \; + C_9H_{19}$

C_9H_{19} -Ph-$(OCH_2CH_2)_x$OH

$\overset{+}{C}H_2CH_2$—⟨benzene⟩—$O (CH_2CH_2O)_x$ H

NPEO$_x$

121($x = 0$)
165 ($x = 1$)
209 ($x = 2$)

$x = 6$ 507 ± 44 $[M + Na]^+ \pm 44$
$x = 8$ 573 ± 44 $[M + H]^+ \pm 44$
$x = 8$ 595 ± 44 $[M + Na]^+ \pm 44$
$x = 9$ 617 ± 44 $[M + H]^+ \pm 44$
$x = 9$ 639 ± 44 $[M + Na]^+ \pm 44$
$x = 10$ 661 ± 44 $[M + H]^+ \pm 44$
$x = 10$ 683 ± 44 $[M + Na]^+ \pm 44$

133–177

⟨benzene⟩—$(OCH_2CH_2)_x \overset{+}{O}$\<H, H\>
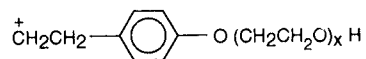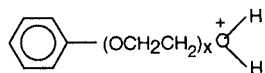
183 ($x = 2$)
227 ($x = 3$)
271 ($x = 4$)
315 ($x = 5$)
359 ($x = 6$)

TABLE 12.7 (*continued*)

Compound	Characteristic m/z ions	m/z ions for n, x
	247 ($x = 1$, $n = 3$)	
	291 ($x = 2$, $n = 3$)	
Poly(ethylene glycols)	$HOCH_2OCH_2OH + Na^+$	$x = 4$ 195 ± 44 $[M + H]^+ \pm 44$
	101	
HO–$(CH_2CH_2O)_x$–H	145 (101 + 44)	
PEG$_x$		$x = 7$ 327 ± 44 $[M + H]^+ \pm 44$
	119 ($x = 1$)	
	163 ($x = 2$)	
	207 ($x = 3$)	
	133 ($x = 1$)	
	177 ($x = 2$)	

TABLE 12.8

MAIN IONS AND RELATIVE ABUNDANCE OBTAINED IN LC-APCI-MS (PI AND NI MODES) AT 20 AND 40 V CONE VOLTAGE FOR THE INJECTION OF 0.2 μg OF TARGET COMPOUNDS [23][a]

M_n	Compound	Relative abundance			
		PI mode		NI mode	
		20 V	40 V	20 V	40 V
184	Benzidine				
	185 $[M + H]^+$	100	100	n.d.	n.d.
	168 $[M - NH_2]^+$	8	68	n.d.	n.d.
212	3,3′-dimethylbenzidine				
	213 $[M + H]^+$	100	7	n.d.	n.d.
	196 $[M - NH_2]^+$	16	100	n.d.	n.d.
	181 $[M - NH_2–CH_3]^+$	n.d.	87	n.d.	n.d.
131	1-methylindol				
	116 $[M - CH_3]^+$	n.d.	100	n.d.	n.d.
	132 $[M + H]^+$	100	89	n.d.	n.d.
179	Acridine				
	180 $[M + H]^+$	100	100	n.d.	n.d.
182	Benzophenone				
	105 $[M - C_6H_5]^+$	25	100	n.d.	n.d.
	183 $[M + H]^+$	100	14	n.d.	n.d.
110	Catechol				
	109 $[M - H]^-$	n.d.	n.d.	100	100
	169 $[M + CH_3COO]^-$	n.d.	n.d.	n.d.	30
139	Nitrophenol				
	138 $[M - H]^-$	n.d.	n.d.	100	30
	108 $[M - NO–H]^-$	n.d.	n.d.	n.d.	100
186	2,2′-biphenol				
	185 $[M - H]^-$	n.d.	n.d.	100	100
108	4-methylphenol				
	107 $[M - H]^-$	n.d.	n.d.	100	100
184	2,4-dinitrophenol				
	183 $[M - H]^-$	n.d.	n.d.	100	30
	137 $[M - NO_2–H]^-$	n.d.	n.d.	20	100
278	Dibutylphthalate				
	279 $[M + H]^+$	27	n.d.	n.d.	n.d.
	277 $[M - H]^-$	n.d.	n.d.	n.d.	64
	221 $[M - C_4H_9]^-$	n.d.	n.d.	n.d.	94
	167 $[C_6H_4(COOH)_2 + H]^+$	70	n.d.	n.d.	n.d.
	149 $[C_6H_4COOCO + H]^+$	100	n.d.	n.d.	n.d.
	77 $[C_6H_5]^-$	n.d.	n.d.	n.d.	100
194	Dimethylphthalate				
	195 $[M + H]^+$	2	n.d.	n.d.	n.d.
	163 $[M + H–2CH_3]^+$	100	n.d.	n.d.	n.d.
220	4-Nonylphenol				
	279 $[M + CH_3COO]^-$	n.d.	n.d.	16	n.d.

TABLE 12.8 (*continued*)

M_n	Compound	Relative abundance			
		PI mode		NI mode	
		20 V	40 V	20 V	40 V
	149 $[CH_3(CH_2)_3C_6H_4O]^-$	n.d.	n.d.	n.d.	100
	191 $[CH_3(CH_2)_6C_6H_4O]^-$	n.d.	n.d.	62	n.d.
	219$[M - H]^-$	n.d.	n.d.	100	n.d.
	149 $[M - (CH_2)_4CH_3]^+$	100	100	n.d.	n.d.
	280 $[M + CH_3COOH]^+$	2	n.d.	n.d.	n.d.
264	Pentachlorophenol				
	263 $[M - H]^-$	n.d.	n.d.	100	100
	229 $[M - Cl]^-$	n.d.	n.d.	30	25
	193 $[M - Cl-HCl]^-$	n.d.	n.d.	10	60
104	2,2-dimethyl-1,3-propanediol				
	87 $[M - OH]^+$	99	n.d.	n.d.	n.d.
	105 $[M + H]^+$	100	n.d.	n.d.	n.d.
	149 $[M + COOH]^+$	n.d.	100	n.d.	n.d.
132	1,1,3,3-Tetramethyl-2-thiourea				
	133 $[M + H]^+$	34	n.d.	n.d.	n.d.
	88 $[M - N(CH_3)_2]^+$	100	100	n.d.	n.d.
150	Ethylbenzoate				
	149 $[M - H]^-$	n.d.	n.d.	100	n.d.
	105 $[C_6H_5CO]^-$	n.d.	n.d.	30	100
141	Isothiocyanate-cyclohexane				
	116$[C_6H_{11}SH]^+$	60	41	n.d.	n.d.
266	Tributylphosphate				
	267$[M + H]^+$	100	18	n.d.	n.d.
	211$[P(OH)_3(OBu)_2]^+$	25	n.d.	n.d.	n.d.
	155 $[P(OH)_3(OBu)]^+$	19	53	n.d.	n.d.
	99 $[M + H-3(CH_2)_4]^+$	n.d.	100	n.d.	n.d.

[a] Experimental conditions: acetonitrile/water (50:50) containing 0.5% of acetic acid at a flow rate of 1 ml/min was used as carrier stream. n.d., not detected.

respectively, for almost all target compounds with low cone voltages. Therefore, this technique allowed to obtain important molecular weight information and $[M + H]^+$ or $[M - H]^-$ ions could be used to confirm molecular weight of the analyte.

Substance-group-specific methods based on LC–MS has been applied for the screening of certain groups of pollutants. For this purpose tandem mass spectrometry (MS–MS) was used to characterize the substance groups, with the help of parent-ion and neutral-loss scans. Identification then followed by means daughter-ion spectra, which were generated by collisionally induced dissociation (CID) using FIA-MS–MS. This protocol showed a good performance for the detection of polyethoxylated surfactants (polyethylene glycols and nonylphenol polyethoxylates) and several sulfates and sulfonates [19]. A rapid method for the direct determination of thiourea in wastewater by APCI tandem mass spectrometry with selected-reaction monitoring was developed. This system acted as a completely

specific detector for thiourea which was determined with a limit of detection of 1 μg/l in tannery effluents [24].

Table 12.9 lists the most common pollutants identified in wastewaters by LC–MS with different interfacing devices. The main part of the listed compounds are highly water soluble and polar or ionic and this type of contaminants are the major contributors for the DOC content of the sample.

In conclusion, the final objective of the presented protocols combining advanced SPE methods followed by GC–MS and/or LC–MS is to achieve a high level of knowledge about the composition and the concentration of pollutants present in contaminated industrial effluents. There is no a unique analytical solution for this purpose and combination of various techniques is recommended in order to determine the most polar and persistent compounds which are often the major contributors to the effluent toxicity.

TABLE 12.9

LISTING OF INDIVIDUAL OR CLASSES OF POLLUTANTS COMMONLY IDENTIFIED BY LC–MS-BASED METHODS IN INDUSTRIAL WASTEWATERS AND THEIR RANGE OF CONTENTS

Compound	Content[a]	Reference
Polyethylene glycols	+ +	[7,19]
Alkylphenol polyethoxylates	+ + + +	[7,19,23]
Aliphatic alcohol polyethoxylates (C10 to C15)	+ + +	[90]
Tributylphosphate	+	[23]
Alkylbenzene sulfonates	+ +	[19,44]
Substituted naphthalene- and benzene- sulfonates	+ + +	[8,45]
Phenolic compounds		
Sulfonated polyphenols	+ + + +	[67]
Chlorinated phenols	+ +	[23,21]
Nitrated phenols	+	[21]
Phenol	+ +	[9]
Catechol	+	[21]
Isocyanate derivatives	+ +	[39,23]
Thiourea derivatives	+ +	[23,24]
Phthalates		
Dialkylphthalates	+ +	[23]
Di(2-ethylhexyl)phthalate	+ +	[23]
Alcohols		
2,2-dimethyl-1,3-propanediol	+ +	[23]
Butanol	+ + +	[9]
Alkyl sulfates and ethoxysulfates	+ +	[43]
Aliphatic hydroxylated carboxylic acids (C1 to C5)	+ + + +	[9]

[a] (+) μg/l; (+ +) 10 μg/l; (+ + +) 100 μg/l; (+ + + +) 1 mg/l.

12.4 RAPID BIOLOGICAL MEASUREMENTS

12.4.1 ELISA tests

Environmental monitoring generally requires to analyse a large number of samples, therefore, there is the need to search low cost, rapid and automated methods for analysis. In the last few years, one of the most developed field testing methods has been enzyme linked immunosorbent assays (ELISA) which have acquired a wide acceptance within the US. As a consequence the USEPA has recently released many official methods based on immunoassay. In example, the method 4010A for the screening of pentachlorophenol (PCP) and the method 4020 for the screening of PCBs among other methods for PAHs, TNT and pesticides using a commercially available immunoassay [46].

The PCP RaPID-ELISA kit has been compared with on-line SPE–LC–APCI–MS for monitoring PCP in industrial effluents and in certified wastewaters. Good correlation between the results obtained by both analytical techniques was observed if PCP concentrations were higher than those of 2,4,6-trichlorophenol which was a cross-reactant for the ELISA determinations. In other cases, ELISA overestimated the PCP determinations due to the high level of cross-reactants present in the sample. This analytical interference due to the limited selectivity of antibodies, commonly referred to as cross-reactivity (CR) is one of the most important aspects to take into account to correctly estimate the final values of the ELISA determinations. Although ELISA measurements need the confirmation of its results by other well-established analytical techniques, it can 'alert' about the presence of relevant contaminants and afterwards appropriate techniques will give the accurate result of all individual analytes.

RaPID-magnetic particle-based ELISA kits for determining carcinogenic polyaromatic hydrocarbons (PAHs) and PCP were also applied to the characterization of industrial effluents [47]. In order to characterize the selectivity of the antibodies, a battery of cross-reactants (structurally similar compounds that compete for the antibody binding sites) was checked. In this case, the selected analytes were obtained from the data reported by the results of the same samples analysed by LC–APCI–MS. Table 12.10 shows the results obtained in the cross-reactivity studies performed using both ELISA tests for the analysis of water samples spiked at the same concentration level as that of wastewaters. The closer recovery to 100%, the higher response. In this sense, all the studied compounds could be detected with the carcinogenic PAHs kit which was giving especially good response for phthalates whereas the Pentachlorophenol kit only showed response for pentachlorophenol and for 4-nonylphenol. Wastewater samples from a petrochemical plant (samples A1, A2 and A3) and from and industrial leachate were analysed giving positive answer with both kits (see Table 12.11). Considering the cross-reactivity results, evidence that the values in Table 12.11 corresponded not only to the target analytes, but also to the cross-reactants present in the wastewater samples was shown. It was observed that although no PAHs were detected by LC–MS, the high response obtained with the carcinogenic PAHs ELISA could be attributed to phthalates that exhibited high cross-reactivity. A good correlation between chromatographic and ELISA determinations could be noticed for the determination of PCP that was detected at a concentration level of 0.4 μg/l LC-APCI-MS, whereas 0.32 μg/l were measured by ELISA. In this case, lower values than the target were observed at higher doses of cross-reactants due to the fact

TABLE 12.10

RESPONSES AND THEIR COEFFICIENTS OF VARIATION (IN PERCENTAGE, $n = 3$) OBTAINED WITH THE PCP AND THE CARCINOGENIC PAHs RaPID ASSAY KITS FOR MEASURING GROUND WATER SPIKED AT THE SAME CONCENTRATION FOUND IN WASTEWATER SAMPLES BY LC–APCI–MS [47]

Compounds	% Recovery	
	Pentachlorophenol RaPID assay	Carcinogenic PAHs RaPID assay
Tributylphosphate	0.11 (5)	37 (2.6)
Dibutylphthalate	–	31 (6.2)
Dimethylphthalate	–	42 (10)
Ethylbenzoate	–	0.16 (8.9)
4-Nonylphenol	0.75 (2.9)	2 (11)
Pentachlorophenol	100 (1)	–
1-Methyl-2-pyrrolidinone	47 (10)	147 (16)
2-Methylbenzenesulfonamide	77 (12)	124 (5.8)
1,1,3,3-Tetramethyl-2-thiourea	0.3 (1.6)	0.68 (3.0)
Isothiocyanate-cyclohexane	–	1.45 (6.9)
2,2-Dimethyl-1,3-propanediol	–	0.93 (2.3)

that the percentage of the cross-reactivity can vary versus the cross-reactant concentrations [47].

Specific detection of the common contaminant 2,4,6-trinitrotoluene (TNT) and its major metabolites was accomplished by means of a capillary-based displacement flow immunosensor. Anti-TNT antibody is immobilized onto the silanized inner walls of a fused-silica capillary using a heterobifunctional cross-linker followed by saturating the capillary with fluorophore-labelled antigen. The target analyte competes for the binding pocket of this antigen, which is detected down-stream. This device permitted limits of detection of 1 ng/l of TNT with good repeatability (4%) and reproducibility (5%) of the system. The capillary immunosensor successfully detected TNT in samples from Superfund cleanup site and the results were comparable to the EPA-certified HPLC method 8330 [48].

Another important point to take into account is the absence of previous treatments like

TABLE 12.11

CONCENTRATIONS AND COEFFICIENT OF VARIATION (IN PERCENT, $n = 6$) OF PCP AND PAHS FOUND IN FOUR INDUSTRIAL WASTEWATERS USING THE PENTACHLOROPHENOL AND CARCINOGENIC PAHs RaPID ELISA KITS [47]

Sample	ELISA PCP (μg/l)	ELISA PAHs (μg/l)
A_1	0.32 (3)	1.88 (8)
A_2	0.45 (10)	1.99 (6)
A_3	7.01 (9)	2.82 (3)
B_1	4.43 (2)	7.8 (8)

filtration or clean-up, showing that these results can only be used as a screening semi-quantitative purpose of the waste water samples and for obtaining a rapid estimation of the contamination of these waste water effluents. In conclusion, although overestimation could be observed due to the absence of treatments in the ELISA determinations and the interferences due to the cross-reactants, ELISA determinations can be used as a measurement of the related contamination present in the waste water samples.

12.4.2 Biosensor detection

Increasing environmental legislation which controls the release and the levels of certain chemicals in the environment has created a need for reliable monitoring of these substances in water. Conventional analytical techniques, although highly precise, suffer from the disadvantages of high cost, the need for trained personnel and the fact that they are mostly laboratory bound. Biosensors because of their specificity, fast response times, low cost, portability, ease of use and a continuous real time signal, can present distinct advantages in certain cases. As a consequence, in the last few years there has been a growing interest in developing new simple, inexpensive and sensitive detection principles capable of monitoring target compounds in environmental samples, e.g., sensors based on biological recognition reaction as biosensors. The biological components used in biosensor construction can in general be divided into two categories: those where the primary sensing event results from catalyst (e.g. enzymes, microorganisms) and those which depend on essentially irreversible binding of the target molecules (e.g. affinity sensors based on antibodies or nucleic acids).

Enzyme-based biosensors have been extensively used and various applications are described using amperometric biosensors for the determination of phenolic compounds, commonly found in industrial discharges. Electrode modification with tyrosinase and laccase for the selective detection of phenols were reported in the literature [49]. A variety of immobilization methods and different electrode materials including surface modification of solid electrodes as well as bulk modification of carbon composite electrodes (e.g., carbon paste [50], graphite epoxy resins [51] and teflon graphite [52]) have been used. A fully integrated screening system for phenolic compounds was developed incorporating on-line solid phase extraction, fractionation and biosensor detection [53]. Two different types of biosensor, solid graphite and carbon paste electrodes (with and without membrane) were tested. Both configurations had the lack of incompatibility with organic solvents and operational instability, respectively. Afterwards the same group developed a new Teflon/graphite composite electrode [52] to solve the above presented problems. It had the advantages of speed, favourable signal to noise ratio, renewability of the sensing surface, rigidity, economy and superior compatibility with organic solvents than the previous mentioned electrode configuration. Nevertheless, few phenolic compounds could be detected by this biosensor unit. In the latest work the range of detectable phenols and selectivity was expanded. For this purpose an amperometric sensor based on immobilization of tyrosinase by means of a polymeric membrane was proposed for detection of a variety of phenolic compounds. The tyrosinase was immobilized into an anionic sulfonated membrane and thereafter introduced into a carbon paste electrode or applied on the tip of a carbon paste electrode. The validation of the biosensor was accomplished by

analysing surface water spiked with a variety of phenols and previously preconcentrated by solid phase extraction (SPE) [54].

An immunosensor was proposed for the analysis of the persistent polychlorinated biphenyls (PCBs) whose analysis by GC–MS is accurate and reliable but tedious and too expensive for screening purposes[55]. This device consisted of a competitive enzyme immunoassay based on alkaline phosphatase using an optimized electrochemical detection for naphthol. The assay was performed in a competitive scheme. A bovine serum albumin (BSA) conjugate, BSA–PCB was the basis for the PCB immobilization procedure. After the competition the amount of IgG anti-PCB that reacted with the immobilized PCB was evaluated using a secondary, alkaline phosphatase labelled, antibody. The detection of alkaline phosphatase was made with naphthyl phosphate and the screen-printed electrodes with differential pulse voltammetry. In another approach, the development of a direct electrochemical immunosensor for the determination of PCBs in water has been reported [56]. The assay was based on the measurement of the current due to the specific binding between PCB and anti-PCB antibody-immobilized conducting polymer (polypyrrole) matrix. The immunosensor exhibited high selectivity for PCBs in the presence of potential interference such as chlorinated anisoles, benzenes and phenols. The highest cross-reactivity measured for chlorinated phenolic compounds relative to Aroclor 1248 was less than 3%. Recoveries of spiked Aroclors 1242 and 1254 from industrial effluent water, rolling mill and seafood plant pretreated water at 0.5 and 50 ng/ml ranged from 103% to 106% as shown in Table 12.12.

In summary, the state-of-the-art of biosensor technology indicates that ideal applications for these devices are continuous monitoring of one class of compounds. For environmental monitoring biosensors would be applicable to situations where the polluting compound was known, or had been identified. They could be used for monitoring factory effluents whose waste product(s) had been identified. Discharge above the legal limits could then be detected on a real time basis. Biosensors could also contribute towards monitoring the progress of clean-up operations after environmental spillages of certain chemicals [57].

TABLE 12.12

MEAN RECOVERIES AND STANDARD DEVIATION ($n = 6$) OBTAINED USING A DIRECT ELECTRO-CHEMICAL IMMUNOSENSOR FOR THE DETERMINATION OF PCBs IN INDUSTRIAL EFFLUENT WATER, ROLLING MILL AND SEAFOOD PLANT PRETREATED WATER SPIKED WITH AROCLORS 1242 AND 1254 AT 0.5 AND 50 ng/ml [56]

Matrix	Spike level (ng/ml)	Measured level (ng/ml)	Recoveries (%)
Industrial effluent water	50.00	53.00	106 ± 6
	0.50	0.52	104 ± 2
Rolling mill pretreated water	50.00	52.00	104 ± 4
	0.50	0.53	106 ± 2
Seafood pretreated water	50.00	52.00	104 ± 6
	0.50	0.50	100 ± 6

12.4.3 Toxicity-based methods

The development and application of toxicity testing is increasing at a rapid rate. A universal monitoring device for toxicity testing is unlikely to be available, but a number of different techniques have been successful in offering some degree of warning of acute levels of toxic material. Toxic compounds exert their biological effect by interacting with cellular structures and functions (membranes, enzymes, energy flux), which are fundamental to all living systems, including bacteria. Most biological toxicity tests are expensive, require large sample volumes and usually take more than 24 h. Consequently, the greatest number of recent studies have dealt within the use of bacterial luminescent assay for toxicity screening and assessment [58] as it produces rapid, reproducible and unambiguous results and it is cost effective. The potential of enhanced chemiluminescence tests as a rapid screening technique for monitoring effluents has been evaluated [59]. There is a wide range of this type of rapid tests available which may be used including Microtox^{TM}, Polytox^{TM}, Rodtox^{TM}, Randox^{TM} and Eclox^{TM}. They all work on the same principle: the enzyme horseradish peroxidase (HRP) catalyses the reaction between the oxidant and a chemical enhancer, to form an enhancer radical. This reacts with the luminol to form a luminol radical which emits light, detected in a luminometer. Light output can be reduced by chemicals which scavenge free radicals, for example antioxidants or inhibitors of the HRP enzyme (e.g. cyanides, amines, phenols and heavy metals). The reduction of light output is usually proportional to the concentration of contaminant present. The mechanics of the reaction are shown in Fig. 12.4. The Eclox test was used for undertaken surveys to examine the reduction in toxicity through different sewage treatment processes on four sewage treatment plants [60]. The mean results for all four plants are summarized in Table 12.13. These results show that primary settlement has little effect on toxicity. However, when assessing toxicity reduction at the biological stage it is the removal of BOD_5 (biological oxygen demand) which has the greater effect on the Eclox output, whilst the ammonia removal results in a further improvement, this is not so significant. In spite of the good performance of this type of test, the determination of ecotoxicity requires the use of different organisms in order to obtain a reliable response of the effect of certain pollutants on aquatic organisms.

The use of a battery of tests seems particularly appropriate for assessing the toxicity of complex effluents due to the number of potential toxicants, the effects of which may be species-dependent and chemical-dependent. In these cases, important attention should be paid to the selection of representative test species, the sensitivity of the tests and the
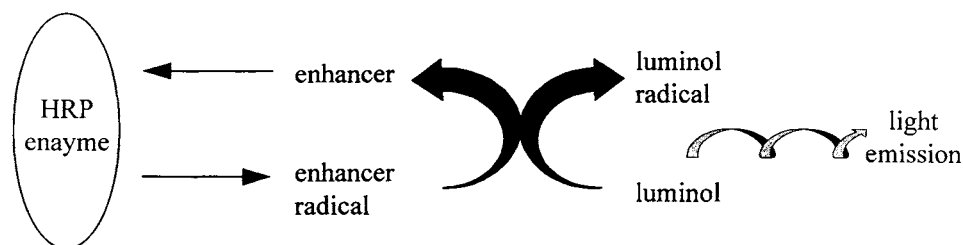
Fig. 12.4. Mechanics of the reaction of rapid toxicity tests based on the principle that toxins present in an effluent interact with the enzyme and inhibit this reaction.

TABLE 12.13

RESULTS GENERATED FROM ECLOX™: ALL SAMPLES FROM SEWAGE TREATMENT PLANTS
WERE TESTED AT 1 IN 40 DILUTION WITH DISTILLED WATER [60]

STP	Sample	% Area inhibition	% Toxicity reduction
A	Crude sewage	89	–
	Settled sewage	84	6.0
	Humus tank effluent	46	49
	Final effluent	27	70
B	Crude sewage	94	–
	Settled sewage	87	7.3
	Asp effluent	45	53
	Final effluent	32	67
C	Crude sewage	94	–
	Settled sewage	78	17
	Humus tank effluent	20	78
	Final effluent	20	79
D	Crude sewage	97	–
	Settled sewage	96	1.0
	Humus tank effluent	30	69
	Nutrient removal plant effluent	26	74
	Final effluent	31	68

simplicity and the costs of the assay. In this sense, 27 landfill leachates were tested on a battery of conventional toxicity tests (microalgae, daphnids, duckweeds) and new microbiotests (rotifers, crustaceans, protozoans, luminescent bacteria) [61]. Table 12.14 lists the test species used, the type of test, the test duration and the endpoints measured and shows that these bioassays comprised acute as well as chronic tests, with exposures ranging from 30 min to 5 days. Leachates of domestic wastes were significantly more toxic than those of pure industrial wastes; the most toxic ones were found for landfills receiving hazardous industrial wastes mixed with domestic wastes. The highest sensitivity was found for the protozoan assay, followed by the crustacean microbiotests. Results suggested that in approximately 90% of the cases, the toxicity of landfill leachates can be assessed by applying a test battery composed of a bacterial assay, a protozoan test and an assay with micro-algae, jointly with one of the following bioassays: higher plants, rotifers or crustaceans. This study revealed that in quite a number of cases, the leachates need to be diluted by more than 10 000 times to make them innocuous for environmental biota [61].

The wide use of the Ames mutagenicity test has long played an important regulatory role in evaluating the toxicity, particularly the carcinogenicity of environmental samples. The test procedure detects reverse mutations in histidine-requiring strains of *Salmonella typhimurium*. Reverse mutations (back to the wild type that does not require histidine) are detected by growth of the bacteria on a histidine-free medium. Mutagenic potency is then generally expressed as the number of revertants on a weight or volumetric basis [62]. Nearly 60% of the genotoxicity studies used this assay due to its ease and cost-effectiveness. In addition, the Salmonella assay has been used more than any other for evaluating

TABLE 12.14

CHARACTERISTICS OF A BATTERY OF TEST-ORGANISMS USED FOR TOXICITY ASSESSMENT OF LANDFILL LEACHATES [61]

Trophic level	Organisms	Type of test	Endpoint	Test duration
Producers	Micro-algae	Conventional	Growth inhibition	5 days
	Scenedesmus subspicatus			
	Duckweed	Conventional	Growth inhibition	5 days
	Lemna minor			
Consumers	Rotifers	Microbiotest	Mortality	24 h
	Brachionus calyciflorus			
	Crustaceans			
	Daphnia magna	Conventional	Mortality	24 h
	Ceriodaphnia dubia	Microbiotest	Mortality	24 h
	Thamnocephalus platyurus	Microbiotest	Mortality	24 h
Decomposers	Bacteria	Microbiotest	Luminescence inhibition	30 min
	Vibrio Fischeri			
	Protozoans	Microbiotest	Mortality	24 h
	Spirostomum ambiguum			

complex mixtures [12]. Consistent with previous investigations using the Ames test [62], the most genotoxic effluents found were those from organic chemical production plants, whereas pulp and paper, metal refining and founding, and petroleum refining facilities also exhibited high potency. Houk [62] categorized the effluents from low to high genotoxicity as illustrated in Fig. 12.5. When considering industrial effluents, the mutagenic potencies in Salmonella ranged from less than 10^2 to 10^{12} revertants per litre of effluent, however the range of potency in single industrial classes most often falls within one order of magnitude. Low values (in the range of 10^2 rev/l) constitute little mutation hazard and conversely, values in the range of 10^{12} rev/l may constitute an extreme mutation hazard. The highest values were found for effluents from furazolidone and nitrofurfural production. In some cases, the mutagenic activity from a single industry could be attributed to specific classes of chemicals. Table 12.15 lists the Salmonella results for 274 compounds (230 carcinogens and 44 noncarcinogens) classified within a chemical class [63]. Another study examined genotoxic loadings (potency × flow rate) revealing that municipal wastewater treatment plant effluent accounted for over 85% of the local increase in genotoxicity in river waters and substances contributing most to the genotoxicity in surface waters are domestic in origin [64]. The coupling of the Salmonella assay with chemical analyses has also been reported. For example, a three-level bioassay-directed chemical fractionation including gel permeation chromatography (GPC) and normal phase (NP) and RP LC [15] was developed. The chemical characterization, directed by Salmonella microsome mutagenicity assay, was carried out by GC–MS. The mutagenic activity recovered among the
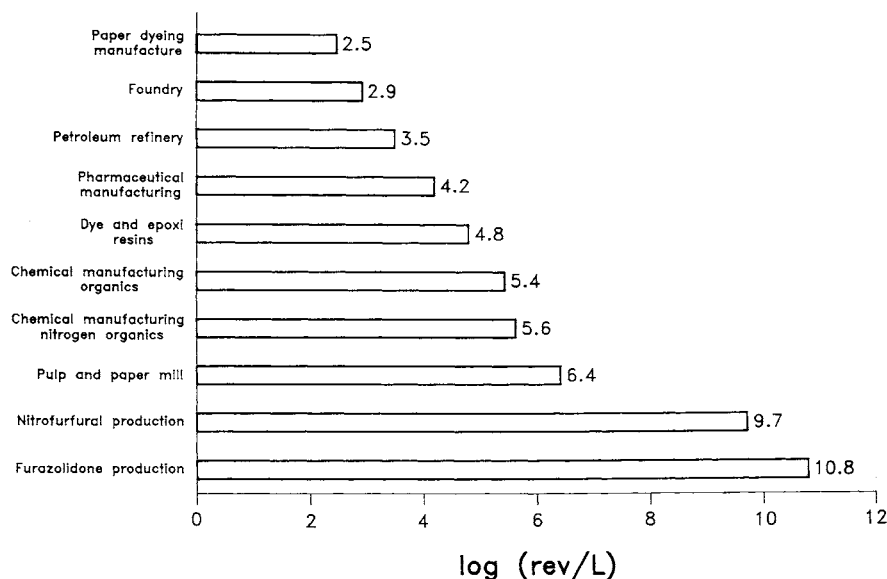
Fig. 12.5. Genotoxic potencies of complex mixtures. The comparison is referenced to the number of revertants histidine-requiring Salmonella bacteria that have undergone reverse mutation per litre of the sample (adapted from [41,82]).

GPC fractions was concentrated (78–98%) in the second fraction which was further fractionated by NP–LC. The highest level of response was recovered in the intermediate-polarity fractions (10–15%); therefore, further fractionation by means of RP–LC was applied.

Given the inconsistency between the results of chemical monitoring for specific substances and genotoxicity measurements, many researchers advocate incorporating genotoxicity assays into environmental monitoring of remediated sites. This is the basis of bioassay-directed chemical analysis, a protocol used to identify specific chemicals or classes of chemicals contributing most to the toxicity of a complex mixture. This method involving SPE of organic material followed by a series of fractionation steps in which toxicity is measured, has been further discussed in Section 2.

Toxicity testing of complex industrial effluents demonstrates that these environmental mixtures contain many unidentified and therefore, unregulated toxicants referred to as nonconventional pollutants (NCPs) representing a risk of unknown magnitude. In order to solve this problem, a strategy for the formulation of toxicity-based consents is studied by the UK Environmental Agency which has been evaluating a range of standard and rapid tests [61]. Toxicity-based consents are already in use in the USA [65] and in Canada on a variety of effluents. The USEPA established toxicity test procedures based on the primary application of a screening test where toxicity was evaluated within 6–8 h, followed by a definitive test if lethality is observed. For the screening tests, a total of 20 organisms are tested for toxicity on nondiluted effluent and on control samples. If the mortality exceeds 10% at any time during the first few hours the effluent is considered to exhibit acute

TABLE 12.15

SALMONELLA BIOASSAY RESULTS FOR ANIMAL CARCINOGENS AND NONCARCINOGENS BY CHEMICAL CLASS [64]

Class no.	Class name	Carcinogens[a]			Noncarcinogens			Totals
		+	i	−	+	i	−	
1	Inorganics	5	1	1			2	9
2	Hydrocarbons	11	3	1				15
3	Ethers	3						3
4	Alcohols	3	9	1			2	15
5	Ketones		2				1	3
6	Aldehydes	1						1
7	Organic acids derivatives			2				2
8	Organic acids		1					1
9	Heterocyclic oxygen compounds	9	3	4			1	17
10	Nitrogen organics	62	17	11	9		5	104
11	Sulfur organics	13	5	4	3	1	4	30
12	Halogen organics	26	10	15	1	1	10	63
13	Other organic compounds	4	1	2	2		2	11
Totals		137	52	41	15	2	27	274

toxicity, therefore a definitive test is initiated. Definitive tests are distinguished from screening tests by the greater length of the test period and the use of multiple concentration of effluent to obtain a toxicity value in terms of a LC_{50}.

12.5 WASTEWATER MONITORING

12.5.1 Petrochemical plant wastewater

A method for the characterization of complex mixtures of mainly polar organic contaminants was set up for its application to the analysis of petrochemical plant discharges [23]. The developed methodology was based on SPE using a SDB-based polymeric sorbent for preconcentration of wastewater followed by LC–APCI–MS characterization. Preliminary recovery studies (see Table 12.3) as well as flow injection analyses (FIA) (see Table 12.8) were performed in order to evaluate the performance of the SPE method and the efficiency of the analytical technique, respectively.

Both NI and PI modes with extraction voltage set at 20 and 40 V were used for real samples in order to detect as many compounds as possible. All compounds were identified by matching retention time and MS spectrum in PI and NI modes with authentic standards. Table 12.16 lists all the compounds observed in the studied samples and their estimated concentration and limit of detection (LOD) for the developed off-line SPE coupled to LC-APCI-MS method. Identification was performed by comparison with the retention time and LC–APCI–MS spectrum of standards. External calibration was used for approximate

TABLE 12.16

CONCENTRATION (RESULTS CORRECTED FOR RECOVERY) AND ESTIMATED LOD OF MAJOR
COMPOUNDS IDENTIFIED IN A PETROCHEMICAL PLANT EFFLUENT USING SPE (WITH A LOAD-
ING VOLUME OF 200 ml) FOLLOWED BY LC–APCI–MS CHARACTERIZATION [23]

Compound	LOD (μg/l)	Conc. (μg/l)
Dimethylphthalate	0.06	0.60
Ethylbenzoate	3.82	51.0
4-nonylphenol	2.91	12.0
Pentachlorophenol	0.37	0.4
1,1,3,3-Tetramethyl-2-thiourea	1.97	39.3
Isothiocyanate-cyclohexane	0.96	11.2

quantification. No internal standard was used for quantification purposes as the broad
range of pollutants detected in the samples made difficult its selection.

Fig. 12.6 shows the negative-ion full-scan LC–MS chromatogram (TIC) and selected
ion monitoring (SIM) LC–MS chromatogram for $m/z = 105$ obtained with extraction
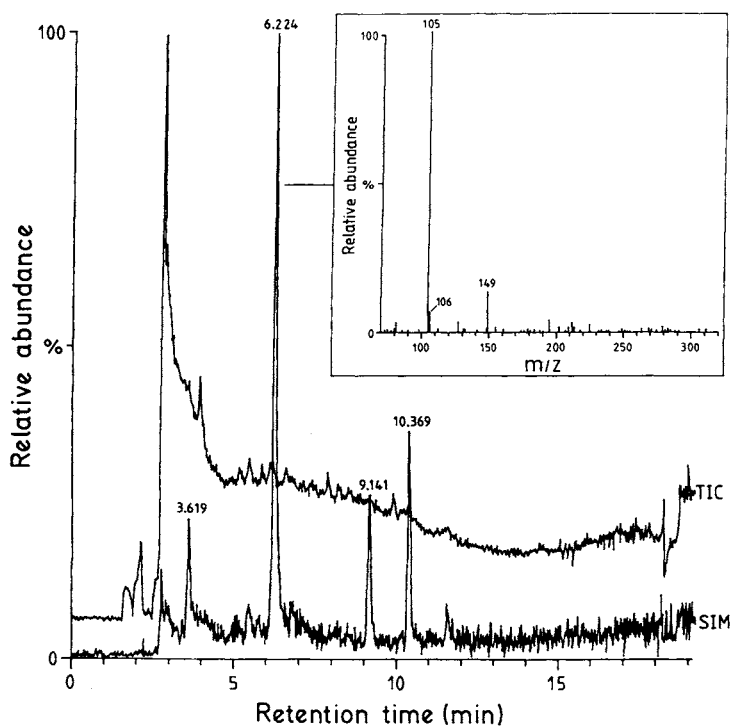
Fig. 12.6. NI full-scan LC–MS chromatogram (TIC) and selected ion monitoring (SIM) LC–MS
chromatogram for $m/z = 105$ obtained with extraction voltage set at 40 V in the injection of 100 μl
of the extract from the preconcentration of 200 ml of wastewater. The spectrum revealed the
presence of ethylbenzoate.

voltage set at 40 V in the injection of 100 μl of the extract from the preconcentration of 200 ml of wastewater. The chromatographic profile obtained in full scan mode showed chromatographic peaks containing unresolved components. Nevertheless, SIM chromatograms provided a more selective method avoiding the presence of interferences. This improved sensitivity allowed detection of ethylbenzoate, frequently used in chemical processes as a solvent. This compound is not particularly toxic (its oral LD_{50} for rats is 2100 mg/kg) and therefore, although the highest concentration level was obtained for ethylbenzoate, this is not of concern. On the contrary, the content of 1,1,3,3-tetramethyl-2-thiourea, which is listed on the USEPA list of toxic substances, is remarkable. Pentachlorophenol is also included in the same list and therefore its presence in the effluent should be taken into account. The rest of pollutants (except ethylbenzoate) are also included in some list regulated by the USEPA, OSHA or EC, and therefore it is necessary to control pollution in order to prevent an alarming level.

The less polar fractions of the same petrochemical plant effluent were analysed by GC–MS [35]. In this case a SSPE procedure was used for preconcentration and fractionation of the sample. Briefly, two different sorbents were used: a C18 phase in series with a SDB-based sorbent, conditioned as described in the previous SPE method. 200 ml of wastewater sample were applied to the C18 cartridge at 5 ml/min and the C18 preconcentrated water was acidified to pH 3.5 if necessary and loaded on the polymeric cartridge at 15 ml/min. The elution step for the polymeric cartridge was performed adding 2×5 ml of methanol at 1 ml/min (fraction D). Differential elution was applied to the octadecylsilica cartridge in order to obtain three extracts containing contaminants with different polarities and functional groups. The eluants system utilized for the stepwise desorption of the analytes from the C18 surface were as follows: eluant A, hexane; eluant B, dichloromethane/hexane (4:1, v/v), eluant C, methanol/dichloromethane (9:1, v/v). Ten ml of eluant A was percolated in two fractions of 5 ml through the C18 sorbent bed at 1 ml/min allowing to obtain fraction A. This operation was repeated with the other two solvents in order to obtain fraction B and C. Total evaporation of the extracts was carried out with a stream of nitrogen. The extracts were reconstituted to a final volume of 1 ml in the appropriate solvent prior to analysis.

The inherent low volatility of several pollutants such as the non-ionic surfactants hampered the application of GC for their analysis. This was overcome by the use of high temperature (HT) capillary columns and a cold on-column injector as an inlet that allows direct deposition of the sample into the column. This injection technique is useful for all types of samples but essential for analysis of thermolabile compounds and of samples with a large (and especially high) boiling point range.

The HT–GC–MS results for the analysis of the less polar fractions of the studied effluents indicated the main advantage of the technique: fast characterization of a great number of different classes of pollutants, including highly functionalized compounds, such as tetraethoxyphenol. Fig. 12.7 illustrates the analysis without any derivatization of a nonylphenol ethoxylates mixture (frequently used as an industrial surfactant) containing between 1 and 7 ethoxy units by HT–HRGC. High resolution of several isomers was afforded in the present chromatogram.

Characterization of the compounds present in the studied samples was carried out by mass spectra interpretation and comparison with library search. Comparison with authentic standards was essential to elucidate the final structures of high-molecular-weight (HMW) compounds that have not been analysed previously by GC–MS. The concentra-
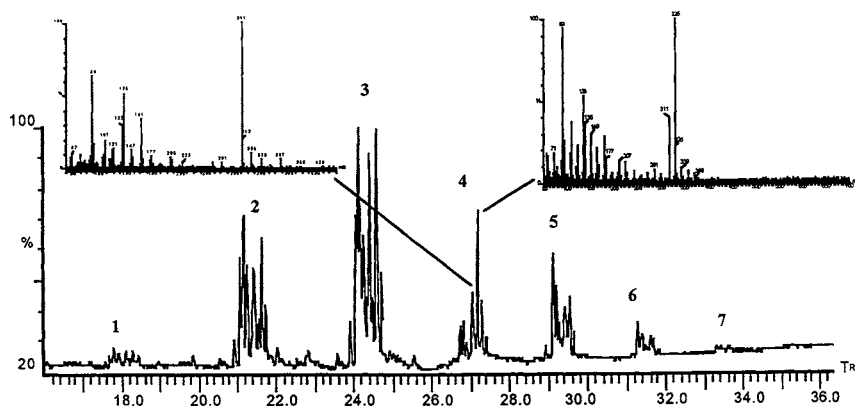
Fig. 12.7. Chromatogram corresponding to the analysis without any derivatization of a nonylphenol ethoxylates mixture containing between 1 and 7 ethoxy units by HT–GC–MS.

tions of organic pollutants were estimated with internal standardization using 4-deuterium-2,6-dichlorophenol as internal standard. More than 50 compounds were characterized revealing the presence of various groups of chemical components in industrial effluents such as phenolic compounds, phthalates, aliphatic carboxylic acids, aromatic carboxylic acids, amines, alkanes and linear aliphatic alcohols. It is noteworthy that some of these pollutants are found in all the samples, indicating that they are commonly used in all industries with no dependence on the type of manufacture. This is the case of phthalates, which are extensively employed as plasticizers. Several phthalates, such as diethylphthalate, butyl-2-hexyl-phthalate, butyl octyl phthalate, benzyl butyl phthalate and diisooctylphthalate were detected. These results were well correlated with those obtained previously by LC–APCI–MS [23]. Referring to the group of phenolic compounds, derivatives from ionol (2,6-di-*tert*-butyl-4-methylphenol) and bisphenol A were detected in most effluents. Recent findings have raised concerns about the health effects of the oestrogen mimic bisphenol A, a compound so widely used in the industrialized world that everyone is exposed to it [66]. Aliphatic carboxylic acids were detected from C_8 to C_{18} with two different origins: the short-chain carboxylic acids of metabolic origin and the long-chain fatty acids of the hides, fatting agents and microbial biomass.

12.5.2 Tannery wastewater

Commonly, analysis of industrial wastewater constituents has to contend with a complex mixture of various organic substances that hamper the extraction and analysis of the compounds under investigation. This is especially true for wastewater from leather production that is known to be heavily loaded with inorganic and organic constituents and its purification is an important task worldwide. Untreated tannery wastewater might contain natural and synthetic dissolved organics up to 3000 mg/l of dissolved organic carbon (DOC) [8,67]. The group of pollutants that are part of DOC includes aromatic compounds, degradation products, polar pollutants and hardly degradable components. A significant fraction of this last group corresponds to non-biodegradable surfactants which

are being used increasingly to remove skin grease in the leather industry and are discharged with wastewater after application.

Major developments have been made in the identification and quantification of organic pollutants in tannery wastewaters by GC–MS and HPLC. GC–MS screening analyses of fractionated wastewater extracts were used for characterization of unknowns [8]. In this work, acidified wastewater samples were solvent extracted and the extracts separated into six fractions by chromatography on silica minicolumns. These fractions were derivatized by bis(trimethylsilyltrifluoroacetamide) to form trimethylslyl derivatives. Analyses were performed on GC–MS in the EI mode. 4-Hydroxybenzoic acid was used as internal standard and quantitative data were therefore estimated. Qualitative assignments were based on spectral library and/or lab-based library. About 200 individual components were determined in tannery effluents like phenolic compounds, low-molecular-weight aliphatic and aromatic carboxylic acids, and a few diethoxylated compounds. Nevertheless, this screening protocol detected only a limited portion of the dissolved organic matter corresponding to an average of 1.2–5.9% of the DOC contents. This is clearly illustrated in the fact that although nonylphenol ethoxylates are of importance in the tanning process, they were not detectable by this protocol. A different approach should be used for characterization of the remaining contaminants. For example, the use of HT–GC–MS [35] (already described in Section 5.1) enabled the determination of nonylphenol tetraethoxylates, although nonylphenols with higher ethoxylation grade were not observed.

LC–MS-based methodology is needed for the characterization of most polar organic compounds with contribution to DOC content of tannery wastewaters. A SSPE method followed by LC–APCI–MS has been developed for the trace determination of non ionic polyethoxylated surfactants in tannery wastewaters [7]. The protocol enabled determination of not only selected analytes but also nontarget compounds, using both NI and PI modes in order to detect as many compounds as possible [11]. Those compounds with available standards were identified by matching retention time and MS spectrum in PI and NI modes. Table 12.17 lists all the compounds observed in the studied samples, the main ions in their spectra and their concentration. Polyethylenegycols (PEGs) and related acidic forms such as different mono- and dicarboxylated PEGs were found in concentration levels ranging from 2.25 to 0.03 mg/l. Alcohol ethoxylated surfactants (ALs) concentration varied between 0.83 mg/l and 0.29 mg/l and nonylphenol polyethoxylated surfactants (NPEOs) were present in the highest level (3.09 mg/l). The use of NPEO is being restricted in some countries due to its slow biodegradation rate and toxicity of its biodegradation products, which have been recently found to be oestrogenic [69]. Fig. 12.8 shows PI full-scan chromatograms showing the presence of nonionic surfactants in different fractions. It should be taken into account that RP–LC has been used and it was therefore possible to separate each individual alcohol ethoxylated homologue. NP–LC is required if alcohol ethoxylated ethoxymers have to be separated [70].

Apart from the target compounds, other different group of organic pollutants were identified. Some phenolic compounds were detected in the most polar fraction, at 6 µg/l and 2.34 mg/l for nitrophenol and 4-chloro-3-methylphenol, respectively. The presence of nitrophenol is attributed to unreacted monomers and 4-chloro-3-methylphenol is use as a preservative agent. Several phthalates was also detected: diethylphthalate and bis(2-ethylhexyl)phthalate) at 35 and 380 µg/l, respectively, in the range of MACs (maximum admissible concentrations) which are recommended on the level of 0.1 to 0.5 mg/l accord-

TABLE 12.17

IDENTIFIED COMPOUNDS, MAIN IONS AND CONCENTRATION (RESULTS CORRECTED FOR RECOVERY) IN UNTREATED TANNERY WASTEWATERS USING SSPE FOLLOWED BY LC–APCI–MS [11]

Retrieved compound	Ionization mode	m/z^c	Fraction	Concentration (mg/l)
PEG$_4$	+	133	D	2.25
		195		
		271		
		291		
		327 ± 44		
Cyclohexane carboxylic acid	+	127	D	n.q.[a]
		139		
2-(Methylthio)benzothiazole (MTBT)	−	136	D	n.q.
		151		
		166		
		181		
		289		
Nitrophenol	−	138	D	0.0065
4-chloro-*m*-cresol	−	141	D	2.34
		143		
Indole acetic acid	+	157	A	n.q.
		175		
Diethylphthalate	+	149	D	2.02
		177		
		223		
2-(Methylsulfonyl)benzothiazole (MsiBT)	+	199	B	n.q.
		275		
		323		
		405		
Monocarboxylate polyoxoethylate (MOE 6) glycol (MCPEG$_6$)	+	133	D	0.75[b]
		151		
		341 ± 44		
MCPEG$_8$	+	429 ± 44	D	0.19[b]
DCPEG$_7$	+	443 ± 44	D	0.25[b]
DCPEG$_9$	+	531 ± 44	D	0.12[b]
AL$_{3,8}$	+	133	C	0.82[b]
		151		
		195		
		413 ± 44		
AL$_{4,8}$	+	581 ± 44	C	0.33[b]
3-(Benzothiazolyl)-benzothiazole-2-thione (BBT)	−	223	D	n.q.
		299		
		381		
NPE$_9$	+	419 ± 44	B	0.049
AL$_{12,4}$	+	133	C	0.83
		151		
		195		

TABLE12.17 (continued)

Retrieved compound	Ionization mode	m/z[c]	Fraction	Concentration (mg/l)
		341 ± 44		
AL$_{13,4}$	+	133	C	1.13
		151		
		195		
		399 ± 44		
AL$_{14,4}$	+	133	C	0.29[b]
		151		
		413 ± 44		
DEHP	+	149	C	0.38
		167		
		279		
		391		
PEG$_4$	+	127	D	0.032
		133		
		177		
		253		
		371 ± 44		
Diethylphthalate	+	149	D	0.035
		177		
PEG$_4$	+	133	D	0.028
		177		
		253		
NPEO$_9$	+	127	B	3.09
		133		
		177		
		271		
		291		
		617 ± 44		

[a] n.q., not quantified due to the lack of standard.
[b] Esimated concentration.
[c] m/z: base peak.

ing to the general toxic and organoleptic indices of harmfulness [71]. Different benzothiazoles derivatives were identified among them: 2-(methylthio)benzothiazole (MTBT), 2-(methylsulfonyl)benzothiazole (MSiBT) and 3-(benzothiazolyl)-benzothiazole-2-thione (BBT). These compounds are widely employed in leather production industry as fungicides instead of chlorophenols. Some of them have mutagenic effects in rats or are allergens and their LC$_{50}$ values against fish have been reported [72], indicating an important contribution to the wastewater toxicity.

Another approach reports the use of LC–ESI–MS for the analysis of polyethoxylated surfactants and their photocatalytic degradation products in tannery wastewaters [73]. For each analyte, single protonated species are observed with the number of ethylene oxide units ranging from 4 to 16. Collisionally activated decomposition of some protonated
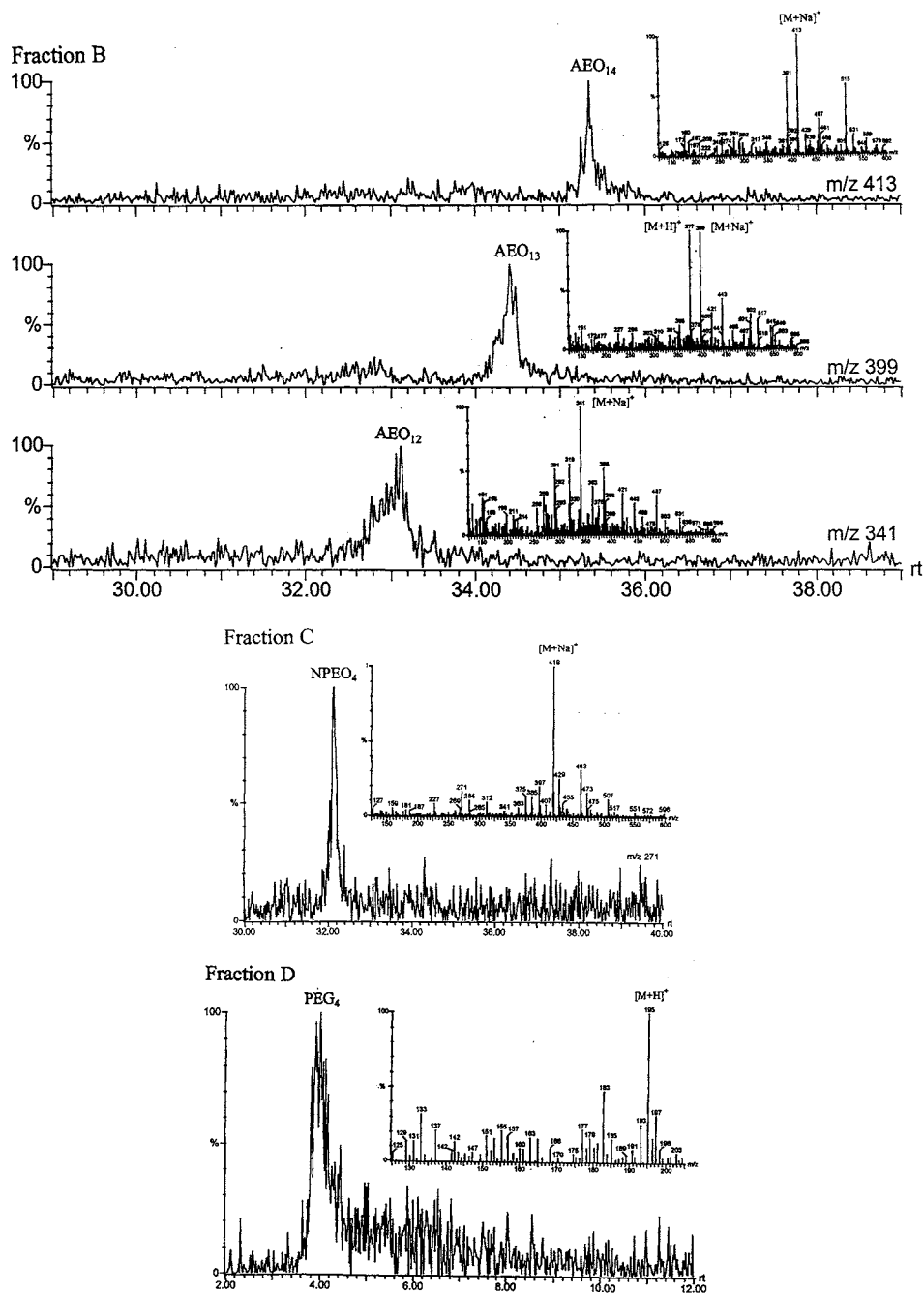
Fig. 12.8. PI full-scan LC–APCI–MS chromatograms of different fractions from a tannery waste-water. The presence of polyethylenegycol PEG$_4$, nonylphenol polyethoxylated NPEO$_4$, and alcohol ethoxylated surfactants C12EO$_3$, C13EO$_4$ and C14EO$_4$ is shown in fractions D, C and B, respectively.

surfactants leads to cleavage of carbon–oxygen bond to give protonated ethylene oxide fragment ions. The surfactants can also be singly ionized in solution by addition of potassium ions. Regarding the photooxidation process, the intermediates observed here are the result of oxidative radical attack and they are neither persistent nor toxic.

12.5.3 Textile wastewaters

Textile wastewaters contain a wide range of non polar and polar compounds, but polar ones are predominant. They comprise substances which are used as auxiliary products in textile production and treatment and are washed out of the textiles having run off with the wastewater. These polar organic pollutants in textile wastewater may give rise to problems due to the fact that they are non-biodegradable and their elimination is incomplete. Moreover, some of the contaminants have a toxic effect on the bacteria applied for wastewater purification [74]. This partly prevents the elimination of these polar compounds from the wastewater by hindering the bacteria in their degradation activity or even killing them. These problems become obvious through the increased content of organic carbon in the textile effluents after water treatment. To help minimize the problems of nondegradability of textile wastewaters, the most complete knowledge of the range of their contents is advantageous. In this sense, separation and identification of polar compounds in textile wastewaters without any derivatization was accomplished by substance-specific detection with MS and/or MS–MS detector [75]. In this case, FIA analysis was employed and separation was based on the different charge ratios (m/z) of the ionized compounds. A requirement for the application of this technique is the use of soft-ionization interfaces so that no fragments are formed which might prevent interpretation of the spectrum. By FIA–MS analysis, information on the molecular mass of the ionized compounds is obtained and for the generation of structure-specific fragments after soft ionization, tandem mass-spectrometry (MS–MS) is needed. Under the total ionization chromatogram (TIC) produced by FIA-MS the 'overview spectrum' is hiding, giving a survey of the molecular- or adduct-ions. Therefore, an impression of the number, and information on the molecular masses of the ionizable constituents is achieved. This is extremely useful when comparison of influent and effluent is performed. However, for other type of applications, time-consuming techniques such SPE followed by LC–MS should be used.

A generic protocol for the determination of a wide range of polar, ionic and highly water soluble organic pollutants was applied to a pilot survey of textile wastewaters [11]. The multiresidual method involved the use of SSPE followed by ion pair chromatography-electrospray-mass spectrometry (IPC–ESI–MS) and by reversed phase-atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS). The extraction procedure consists in SSPE using an octadecylsilica cartridge in series with a polymeric one where anionic analytes were trapped and eluted with a solution 5 mM in triethylamine (TEA) and 5 mM in acetic acid/methanol (1:9, v/v). Fractionated extracts were obtained from the C18 cartridge by differential elution (see Section 6.2). This protocol enabled average recoveries ranging from 72% to 103% (see Fig. 12.1) together with group fractionation as aromatic compounds were mainly collected in the first extract eluted from the C18 cartridge, medium polar compounds were included in the second one and highly polar

compounds were eluted from the polymeric sorbent. Furthermore the use of SSPE led to the elimination of interferents present in complex water matrices.

LC–MS was used for characterization of textile wastewaters. Detection of nonionic pollutants was performed by means of an APCI interface, whereas anionic ones were determined by ESI-MS in NI mode. For example linear alkylbenzene sulfonates were determined at a cone voltage of 80 V using the peak at m/z 183 whose corresponding ion was $[CH_2=CH–C_6H_4–SO_3]^-$ as diagnostic ion. When analysing napthelene and benzene sulfonates (NPS and BS) by IPC-ESI-MS with fragmentor voltage set at 150 V, it has been observed common losses of the groups HSO_2 or HSO_3, therefore the ions corresponding to the structures $[M - HSO_2]^-$ or $[M - HSO_3]^-$ are diagnostic ions. However, when applying lower voltages (80 V), the mass spectra is dominated by the presence of the $[M - H]^-$ or $[M - 2H]^{2-}$ ions for mono- and disulfonate compounds, respectively.

After checking the performance of the developed multiresidual method, it was applied to the identification and quantification of textile wastewaters. Different groups of compounds such as non ionic surfactants (glycol-, nonylphenol- and alcohol polyethoxylates), anionic surfactants (alkylbenzenesulfonates), benzene- and naphthalene-sulfonates,) and related industrial compounds many of them highly water soluble were retrieved. Table 12.18 lists all the compounds observed in the studied samples, the main ions in their spectra and their average concentration in the different analysed samples. Polyethylene glycol (PEG), polyethoxylated decylalcohol (AEO_{10}) and 2-naphthalenesulfonate (2-NPS) were found as major pollutants in textile wastewaters with concentrations up to 1.2, 3.5 and 2.4 mg/l, respectively. Regarding to the group of anionic compounds, Fig. 12.9 shows the (NI) ESI-MS chromatograms corresponding to the most polar fraction and to a medium polarity fraction where aromatic compounds are recovered. In fact, linear alkylbenzene sulfonates (LAS) were present in this fraction at concentration levels ranging from 14.2 µg/l for C10LAS to 39.1 µg/l for C11LAS. Higher concentration levels were found for some benzene- (BS) and naphthalene-sulfonates (NPS) which are used in the textile industry as dye bath auxiliaries. In summary, it was shown that identification of non ionic and anionic surfactants and aromatic sulfonates in wastewaters was feasible by applying the combination of LC–APCI–MS, LC–ESI–MS and IP–ESI–MS with prior tailor-made SPE procedures.

The important amounts of NPS and BS in textile wastewaters have encouraged the development of analytical strategies for their specific determination. For example, the use of SPE followed by IPC with ultraviolet (UV) detection has been reported [68]. In this case a graphitized carbon black with positively charged oxonium groups was used as sorbent due to its high capability to selectively adsorb aromatic anions mainly by an anion exchange mechanism. The low octanol/water partition coefficient ($\log P_{ow} < 2.2$) and the reduced biodegradability of substituted BS and NPS indicate that they are not easily eliminated in sewage treatment plants and therefore, their presence in industrial discharges should be controlled. Furthermore, toxicity of this group of compounds to different target organisms like bacteria, algae and fish has been reported [76].

12.5.4 Pulp mill effluents

The pulp industry utilizes large amounts and a wide variety of chemicals in the operation of various chemical processes, discharging large volumes of effluents. In

TABLE 12.18

IDENTIFIED COMPOUNDS, MAIN IONS AND AVERAGE CONCENTRATION IN TEXTILE WASTE-WATERS USING SSPE–LC–APCI–MS, LC–ESI–MS AND IP–ESI–MS [11]

Retrieved compound	Analytical procedure	Ion. mode	*m/z*	Conc. (μg/l)
PEG$_2$	SSPE–LC–APCI–MS	PI	101	1210
			106	
MCPEG$_2$	SSPE–LC–APCI–MS	PI	133	830
			151	
			187 ± 44	
C10LAS	SPE–LC–ESI–MS	NI	183	14
			297	
C11LAS	SPE–LC–ESI–MS	NI	183	39
			311	
DBP	SSPE–LC–APCI–MS	PI	149	226
			205	
			256	
C12LAS	SPE–LC–ESI–MS	NI	183	35
			325	
C13LAS	SPE–LC–ESI–MS	NI	183	22
			339	
AEO$_{10}$	SSPE–LC–APCI–MS	PI	133	3513
			177	
			151	
			401 ± 44	
DEHP	SSPE–LC–APCI–MS	PI	149	173
			167	
			279	
			391	
3-NitroBS	SPE–IP–ESI–MS	NI	80	132
			156	
			202	
1-Hydroxy-4-NPS	SPE–IP–ESI–MS	NI	80	21.4
			159	
			223	
1-Amino-7-NPS	SPE–IP–ESI–MS	NI	80	2.4
			158	
			222	
2-NPS	SPE–IP–ESI–MS	NI	80	2377
			143	
			207	

some cases the pulp is brightened or bleached by a variety of chemical techniques which result in the discharge of a complex mixture of contaminants. Pulp industry effluents usually contain resin and fatty acids, a variety of chlorinated organic compounds such as chlorophenols, chlorocathecols or guaiacols and volatile compounds including the group designated as total reduced sulfur compounds such as methylmer-

Fig. 12.9. (a) NI full-scan LC–ESI–MS chromatogram (extracted ion $m/z = 183$) revealing the presence of C10LAS, C11LAS, C12LAS and C13LAS as shown in the spectra. (b) NI full-scan IPC–ESI–MS chromatogram (extracted ion $m/z = 207$) obtained with fragmentor voltage set at 80 V and 150 V confirming the presence of 2-naphthalenesulfonate.
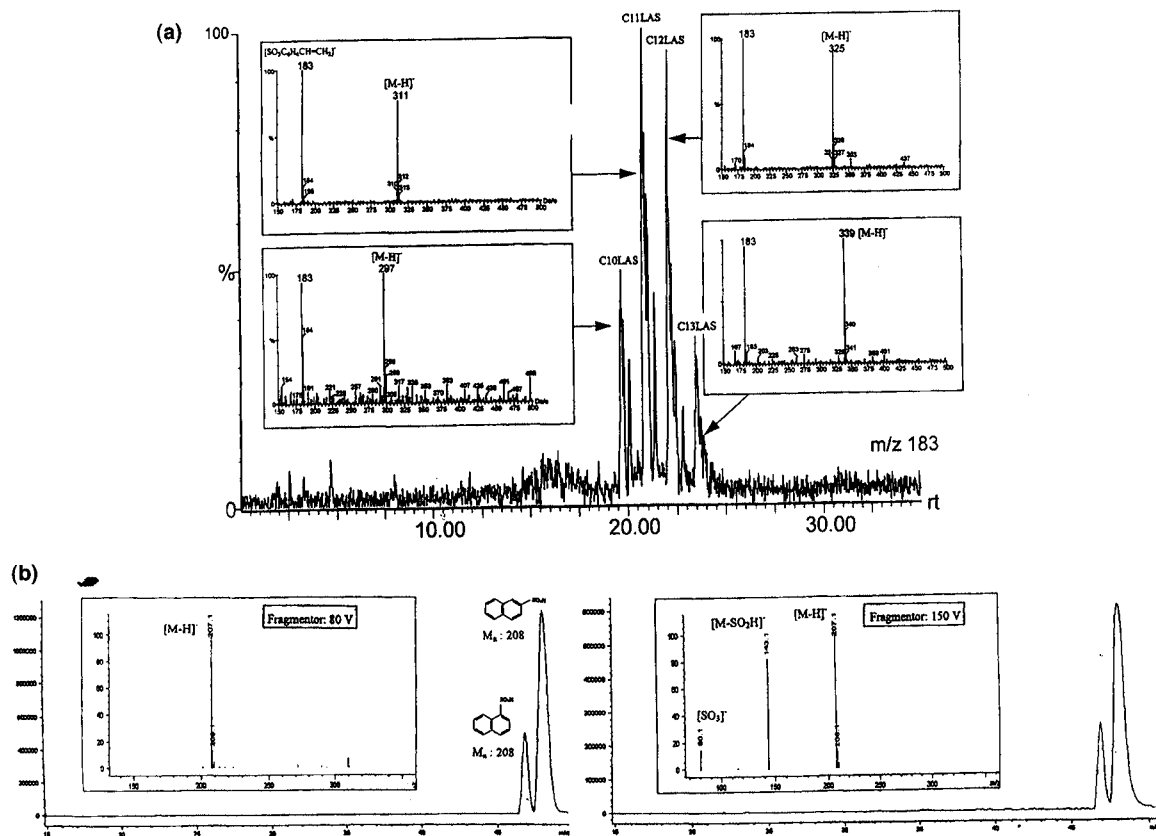
captants, or methyldisulfides. Tetrachlorocathecols and tetrachloroguaiacols were reported in pulp effluents in the range of 2–240 μg/l and 1–120 μg/l, respectively, depending on the type and plant design and on the applied water treatment [77]. The only effluent treatments which are widely used by the pulp industry involves primary treatment, consisting in elimination of suspended solids and secondary treatment with microbiological oxidation of fermentable dissolved organic constituents [78]. However, there is evidence that some organic compounds are not completely eliminated by this process, as was pointed out by the USEPA [79].

Analysis of pulp mill effluents before and after treatment for monitoring phenolic compounds was carried out using a methodology based on on-line SPE with polymeric sorbents followed by LC–APCI–MS [21]. Results for LC–APCI–MS experiments in full scan mode showed the presence of 2-chlorophenol, 2,4-dichlorophenol, 4-nitrophenol and catechol in both cases (influent and effluent) but no traces of high chlorinated phenols as well as other priority phenols were found. Values obtained for untreated water varied between 2.5 and 27.3 μg/l. Although these levels were reduced by the treatment, completely removal did not occur. Surprisingly, catechol was found in the effluent but not in the influent thus meaning it originated during the treatment of wastewater.

The levels of fatty acids, resin acids, degraded resin hydrocarbons and chlorophenolic substances at points above and below the final discharge of a pulp mill have been investigated [80]. The analytical approach used to carry out this survey consisted on LLE followed by GC–MS after derivatization of the extracts.

Another work [81], reports chlorination by-products from biologically treated thermo-chemical (TMP) chemithermomechanical (CTMP) pulp mill effluents. The organic material remaining in this type of effluents is capable of reaction with chlorine under drinking-water treatment conditions, producing compounds of concern. Specifically increased concentrations of chloroform, trichloroacetic acid, and 2,4,6-trichlorophenol were associated with natural waters containing CTMP mill effluents. It was finally concluded that the relative effect of a TMP or CTMP mill effluent on a downstream drinking water treatment plant will thus depend on the available dilution (flow conditions) and the amount of natural organic matter present in the receiving water.

Capillary gas chromatography, electron impact and negative ionization mass spectrometry, retention indices, flame ionization and halogen-specific detectors were used for analysis of discharges from a softwood pulping and kraft paper operation [25]. Many compounds were identified and were derived from natural products released from wood during pulping [82]. Among them: guaiacols, syringaldehydes, dehydroabietic acids, thiophenes and isoborneol.

Using a similar analytical approach, the laboratory generated spent bleach liquor from the chlorination and caustic extraction stages of bagasse and wheat straw pulps have been analysed [83]. A number of chlorinated derivatives of phenols, catechols, guaiacols, syringaldehydes, resin acids as well as unchlorinated saturated and unsaturated fatty and resin acids have been identified. The quantities of various chlorophenolic compounds and resin and fatty acids present in the effluents was compared with reported [96]LC$_{50}$ values [84]. The concentrations of 2,4,6-trichlorophenol and pentachlorophenol were observed to be higher than the reported lower limits of [96]LC$_{50}$. On the contrary, observed values of concentration of resin and fatty acids were lower than the reported toxicity values. It

should be taken into account that $^{96}LC_{50}$ values describe the toxicity of a particular compound when present alone. However, when a number of toxic compounds are present, interfering effects may be observed. Hence, it was concluded that the untreated spent bleach liquor can be considered toxic in nature, therefore, such effluents should not be discharged without treatment.

Some recent works, reports the presence of contaminants with estrogenic properties in paper mill effluents. An analytical method was developed for the quantitative determination of nonylphenol polyethoxy carboxylated (NPEC) metabolites of NPEO surfactants in paper mill effluents [85]. Strong anion exchange SPE disks were used for the NPEC isolation from aqueous samples, and NPEC elution from the disk was simultaneously combined with derivatization. Extracts were analysed by GC–MS in positive ionization mode with chemical ionization. The total concentration of NPEC in a group of paper mill effluents ranged from below detection (0.2–2 μg/l) to 1300 μg/l. This type of discharges typically contained less than 100 μg/l NPECs. The NPEC concentrations measured were below those associated with acute and chronic fish toxicity [86]. Another approach employs an in vitro recombinant receptor/reporter gene assays to examine pulp and paper mill black liquor and effluent for estrogenic, dioxin-like and anti-oestrogenic activities [87]. It was estimated that black liquor contains 4 ± 2 ppb 'oestrogen equivalents', while negligible estrogenic activity was observed in a methanol-extracted pulp and paper mill effluent fraction. Black liquor and effluent fraction contain 10 ± 4 ppb and 20 ± 6 ppt of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin equivalents, respectively. Moreover, the effluent fraction extract exhibited significant anti-oestrogenic activity. These results demonstrate the utility of bioassays for characterization of industrial effluents and suggest that the effects observed in fish exposed to pulp and paper mill effluent may be due to unidentified ligands not detected by conventional chemical analysis due to the lack of appropriate standards.

12.5.5 Ammunition plants

Residues of high explosives are a significant pollution problem at US and European military facilities. In fact, nitroaromatic and nitroamine explosives along with several of their manufacturing impurities and environmental degradation products have been observed in groundwater at a number of U.S. Army installations. A SPE method followed by LC–UV with confirmatorial column was applied to the analysis of several samples from military facilities revealing the presence of 26–318 μg/l of hexahydro-1,3,5-trinitro-1,3,5-triazine (HMX), 82–618 μg/l of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), 9–284 μg/l of 2,4,6-trinitrotoluene (TNT) and 9.6–239 μg/l of 4-amino-2,6-dinitrotoluene (4A-DNT) [88]. Other authors used mass spectrometry and tandem mass spectrometry coupled by a thermospray interface to a LC system equipped with a photodiode array detector for the determination of nitroaromatic explosives and their degradation products in unsaturated-zone water samples in the USA [89]. Using this approach, average concentrations of 12 μg/l, 18 μg/l, 23 μg/l and 65 μg/l were obtained for 1,3,5-trinitrobenzene, 3,5-dinitroaniline, TNT and 4A-DNT, respectively. A GC–MS methodology enabled identification of alkylated benzenes and naphthalenes present in the mg/l range in military and other federal installations [25].

12.6 CONCLUSIONS AND FUTURE DEVELOPMENTS

A discussion on the application of some classic analytical methods such as LLE followed by GC–MS to the analysis of organic pollutants present in industrial waste discharges has been presented. The good performance of alternative extraction methods such as SPE or SPME has permitted a great increase in its use and acceptance. In reference to chemical analysis technology, GC–MS has been established as a routine and reliable technique and the development of LC–MS has led to the establishment of new LC–MS-based analytical techniques for the determination of polar analytes in wastewaters.

In spite of the great development of analytical devices, characterization of industrial effluents is a complex issue and most of the organic content in wastewaters still remains unidentified. As a consequence, unknown environmental risk of industrial emissions exists. Therefore, there is the need of combining chemical analysis based on extraction and fractionation of the extracts followed by LC–MS or GC–MS with toxicity evaluation with a battery of reliable tests.

Furthermore, some contaminants are commonly very polar, with reduced biodegradability are may not be eliminated in mechanical-biological treatment plants and thus end up in the aquatic environment. Consequently, efforts on the establishment of suitable procedures for the determination of polar, ionic and highly water-soluble pollutants have been made in the last few years. These protocols involved the use of LC coupled to MS mainly by atmospheric pressure ionization (API) interfaces whose great development has provided rugged and reliable interfaces. Although API techniques do not offer a complete EI spectrum, the combination of PI and NI modes at different cone voltages allows enhancement of structural information. Problems responsible for incomplete characterization of these samples by LC–MS included the lack of standards necessary to identify and quantify all the peaks in the chromatogram.

Future work will include the gradual build-up of a laboratory mass spectra library obtained with the experimental conditions of the methodology established for the analysis of wastewaters. The development of rapid ELISA and biosensors for the analysis of wastewaters will improve target analysis in complex industrial matrices. Much effort should be focused on the toxicity studies of some polar pollutants that have been recently found in high amounts in industrial discharges. This is the case for naphthalene- and benzene-sulfonates, whose presence in wastewaters has not been reported before due to the unavailability of suitable techniques for its analysis.

In summary, the main goal in wastewater analysis is the complete characterization of the discharges in order to totally identify TOC contributing to the toxicity of the sample. In this sense, new analytical devices or improvements of established techniques will contribute to increase the knowledge on the organic content of the sample. Combination of LC–MS–MS and synthesis of authentic standards to unequivocally identify unknowns is continuously needed.

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Chapter 13

Mine waste characterization

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CONTENTS

13.1 MINE WASTE

Mine waste, often called mine spoil, is the geologic material discarded during mining [1]. Mine waste includes waste rock from below ground mining and surface or strip mining. Overburden is the geologic material on top of strata that are being mined in surface or strip mine types of operations (e.g., waste rock on top of coal seams). Tailings are the waste geologic material from mineral processing operations. Thus, mine spoil, overburden, and tailings are all mine waste [1].

The geologic material in mine waste may be igneous, metamorphic, or sedimentary in origin. Igneous, sedimentary, and metamorphic rocks are classified by mineral composition and texture [2]. A wide range of different rock types may be found in mine waste. The composition of mine waste depends not only on the geologic environment which waste

rock came from, but also on the type of mining that produced the waste rock. Some examples include sedimentary rock of different particle sizes from sand and gravel pits, mudstones and shales from phosphate rock mining, spoils containing pyrite from strip mining of coal seams, and waste rock containing metal sulfides from precious metal mines. This range of waste rock types presents numerous challenges to the analyst trying to provide physical, chemical, and mineralogical characterization of the mine waste.

Mine waste characterization data are needed to support a reclamation plan to repair the damage caused to the environment by mining operations. Mine waste is usually an inhospitable environment for re-establishment of native plant communities, which is a necessary step in a successful reclamation effort. The physical and chemical properties of many mine wastes are far less than optimal for plant growth (Table 13.1). Thus, remediation treatments are usually necessary for the mine waste to support plant communities.

A fortune could be spent collecting comprehensive and detailed physical, chemical, and mineralogical data from mine wastes prior to initiating a reclamation plan. Fortunately, the more important parameters that characterize the waste with regard to undesirable physical properties, nutrient levels, and potentially toxic levels of some elements (e.g., heavy metals) comprise a more manageable list (Table 13.2).

Various analytical methods have been developed to measure the parameters listed in Table 13.2. The many references for these methods should be consulted for details on principles, supplies, equipment, reagents, standards, procedures, and quality control [3–5]. These references are among the main sources of analytical methods for mine waste analysis in the geologic, soils, and environmental literature. The methods presented in this chapter were compiled from these sources as well as individual journal articles and are a representative cross-section of some of the more important methods. The focus of the chapter is on the analytical methods themselves and not on interpretation of the results, although some general interpretations will be given. For more detailed information on

TABLE 13.1

PHYSICAL AND CHEMICAL PROPERTIES OF MINE WASTES THAT MAY LIMIT PLANT COMMUNITY RE-ESTABLISHMENT

Physical properties
Low water holding capacity
Excessively fast or slow permeability
Large coarse fragment content

Chemical properties
Arid-region mine wastes:
  Excess soluble salts (salinity)
  Excess levels of exchangeable sodium (sodicity)
  Excess alkalinity (pH > 8.8)
  Low levels of organic matter, nitrogen, phosphorus, potassium, and other nutrients
  Potentially toxic levels of arsenic and selenium
Acidic mine wastes with metal sulfides:
  Excess acidity (pH < 4)
  Low levels of organic matter, nitrogen, phosphorus, potassium, and other nutrients
  Potentially toxic levels of metals (e.g., Ni, Cu, Zn)

TABLE 13.2

PHYSICAL AND CHEMICAL PROPERTIES OF MINE WASTE THAT SHOULD BE MEASURED FOR A
COMPLETE ROUTINE CHARACTERIZATION

Physical properties
Water content
Bulk density
Particle size distribution (e.g., sand, silt, and clay)
Water retention curve

Chemical properties
Electrical conductivity and sodium adsorption ratio (SAR) of a saturation extract
pH of a 1:1 mine waste - water suspension
Carbon (organic matter, total organic carbon, carbonates)
Nitrogen (total, organic, inorganic N species)
Extractable phosphorus
Exchangeable cations (Na, K, Mg, Ca, transition metals) and cation exchange capacity
Extractable metals (e.g., V, Cr, Mo, Mn, Fe, Co, Ni, Cu, Zn, Cd, Pb) and metalloids (e.g., As and Se)
Lime requirement, sulfur forms, and acid–base accounting

interpreting mine waste analysis, developing a reclamation plan, and applying reclamation
techniques, consult the many references available on this subject [6–10].

13.2 SAMPLING

Perhaps the most difficult task of all is obtaining a representative sample(s) of the mine
waste to be treated. The spatial heterogeneity of mine waste found at most sites makes
devising a reliable sampling plan problematic. Sampling plans that have been developed
for soils are often applicable to mine waste [11].

The three basic types of sampling plans normally used are simple random, stratified
random, and systematic [11]. Simple random sampling in which samples are taken
randomly and independently of each other from a mine waste pile or an area to be treated
for reclamation produces unbiased samples. However, because of the large spatial hetero-
geneity that is frequently encountered, this type of sampling plan is usually not useful for
mine waste.

In stratified random sampling, the mine waste is subdivided into relatively more homo-
geneous strata prior to sampling. The subdivisions could be spatially horizontal and/or
vertical (depth) depending on the objectives of the sampling plan. Samples from within
each strata, are then collected at random. This sampling plan is appropriate where rela-
tively uniform areas can be delineated prior to sampling. These delineations might be
based on topography or some visual characteristic of the mine waste.

In systematic sampling, transects or grids are used to delineate the sampling points. This
type of sampling is often the best for mine waste, particularly if no prior knowledge of the
characteristics or spatial heterogeneity of the waste is known. The results of such a
sampling plan can be used to map the spatial heterogeneity of the mine waste.

To reduce the total number of samples, but still capture the variability that is inherent at

most sites, composite sampling is often a good choice. With this approach, individual samples from a given area are composited to produce a single sample from that area. Each area to be sampled is represented by similarly collected composite samples. For this approach to be successful, each individual sample must contribute the same amount to the composite sample.

The systematic and composite sampling methods are being used at the Summitville, CO, USA Superfund site [12,13]. The approximately 300-ha site is being characterized by one composite sample per 0.4 ha. Each composite sample consists of four samples, one per 0.1 ha.

Sampling tools are dictated by the type of mine waste, the objectives of the sampling program, and the analyses to be run. If intact cores are needed for bulk density and other physical property determinations, then a hammer or mechanically driven coring tool must be used. If a disturbed sample is allowed, then hand or mechanically driven bucket augers may be used. Power drilling equipment is usually only needed if the mine waste is mostly rock or if relatively deep cores are needed. Sample containers need to be chemically inert and can include wide-mouth plastic bottles, zip-lock bags, whirl-pack bags, and plastic-lined heavy paper bags among others. Sample amounts depend on the analyses to be conducted. To allow for inert coarse fragments that may be discarded, sample splitting, and replicate analysis, generally a 1–2 kg sample is sufficient.

13.3 SAMPLE PREPARATION

Many mine waste samples contain moisture and need to be dried prior to any analysis. We recommend gentle air-drying at room temperature for most chemical analyses recognizing that even air-drying can alter some of the chemical properties of soils [14] and probably of mine waste as well. Oven-drying is usually only appropriate if total chemical composition is to be determined because it can drastically alter some of the chemical properties of samples. If water content at the time of sampling is needed, then the sample must be placed in a sealed container at sampling to prevent moisture loss. The water content of a representative subsample can be determined gravimetrically with oven drying [15]. If intact cores for bulk density measurements are taken, these cores can also be used to determine water content.

Following air-drying, the sample should be sieved through a 2-mm plastic or stainless-steel sieve to remove coarse fragments. Either hand or mechanical sieving is satisfactory depending on the sample workload. The <2-mm size fraction is retained for analysis. If the coarse fragment content is to be determined, then the >2- and <2-mm fractions are weighed. The >2-mm fraction (coarse fragments) should only be discarded if it is known to be relatively inert. In some types of mine waste, the coarse fragments contain significant quantities of metal sulfides that eventually undergo physical and chemical weathering, and thus contribute acidity, metals, and sulfur to the surrounding environment. If this is the case, then the >2-mm fraction should be crushed with a rock crusher and analyzed separately from the <2-mm fraction.

If digestion methods are to be used in a total analysis of the mine waste, samples can be ground to pass smaller size fraction sieves (e.g., 0.5, 0.25, 0.125 mm, etc.) to improve digestion efficiency and precision. In general, as sample amount decreases, particle size

must also decrease to achieve reproducible results. For sample amounts of 200 mg or less, the sample should be ground to pass a 0.125-mm sieve. Grinders with ceramic or nonmetallic grinding parts are preferred because they do not introduce metal contaminants into the sample.

Sometimes the analyst will want to determine the properties of specific size fractions (e.g., 2000–1000, 1000–500, 500–250, 250–125, 125–53, 53–20, 20–2, 2–0.2, < 0.2 μm). In such cases, particle size separations need to be done prior to any analyses [16].

13.4 PHYSICAL PROPERTIES

Bulk density, particle size distribution, and water content are probably the most useful physical property measurements for mine wastes. Water retention curves and hydraulic conductivity measurements, although certainly useful, are often not collected for routine characterization purposes. Water retention characteristics can be estimated from a particle size analysis [17]. If a particular mine waste is suspected of having undesirable physical properties that would severely limit plant growth (e.g., low water holding capacity because the mine waste is very coarse textured or very low permeability because the mine waste has a high montmorillonite clay content), then a water retention curve and hydraulic conductivity measurements would be useful.

13.4.1 Bulk density and water content

Amounts of mine waste remediation amendments (e.g., lime, fertilizer) are typically based on analytical data from known sample weights. Bulk density (weight/volume) measurements are important because, in the field, it is more convenient to apply treatments per unit area or volume of mine waste. Knowing the bulk density of the mine waste allows conversion from one type of units to another.

The core method of determining bulk density is often unsuitable for mine waste because the coarse fragment content is too high for this method to be used effectively. For mine wastes high in coarse fragment content, the excavation method is usually a better choice.

In the core method, an intact soil core of known volume is taken by a hammer or mechanically driven core sampler [18]. The core is weighed, oven-dried at 105°C, and re-weighed. The wet and dry weights are used to calculate water content at the time of sampling:

$$\theta_{dw} = \frac{\text{wet weight of sample (g)}}{\text{oven-dry weight of sample (g)}} - 1 \qquad (1)$$

where θ_{dw} is the gravimetric water content (dry-weight basis).

The oven-dry weight and core volume are used to calculate bulk density;

$$\rho_b = \frac{\text{oven-dry weight of sample (g)}}{\text{volume of core (g)}} \qquad (2)$$

where ρ_b is the sample bulk density (g/cm^3).

The relationship between volumetric and gravimetric water content is given by

$$\theta_v = \frac{\rho_b}{\rho_w} \theta_{dw} \tag{3}$$

where θ_v is volumetric water content (cm^3/cm^3) and ρ_w is water density.

In the excavation method, a 12×12 cm (approximately) hole is excavated and the mine waste is placed in a sample bag for return to the laboratory [18]. The exact volume of the excavation is measured by the sand funnel or balloon technique. In the laboratory the mine waste sample is weighed, oven-dried at 105°C, and re-weighed. Water content and bulk density are calculated in a similar manner as the core method.

13.4.2 Particle size

Determining the mass of coarse fragments (>2 mm) in a mine waste sample has already been covered in Section 13.3 above. In this section a method for determining particle size distribution (sand, silt, and clay fractions) of the <2-mm fraction is given (Table 13.3). The method uses a combination of hydrometer readings and wet and dry sieving to determine particle size distribution [16].

The hydrometer readings are used to determine the concentration of mine waste in the hexametaphosphate (HMP) dispersing solution at each settling time based on Stoke's law:

$$C = R - R_L \tag{4}$$

where C is the mine waste concentration in suspension (g/l), R is the hydrometer reading in the sample suspension (g/l), and R_L is the hydrometer reading in the HMP reference solution. A summation percentage (P) is computed for each settling time:

$$P = \frac{C}{C_0} \times 100 \tag{5}$$

where C_0 is the initial oven-dry sample weight (g). The effective hydrometer depth (h') in cm is given by

$$h' = -0.164R + 16.3 \tag{6}$$

A parameter B is calculated:

$$B = \frac{30\eta}{g(\rho_s - \rho_l)} \tag{7}$$

where η is the HMP solution viscosity (g/cms) at the measured temperature of the solution, g is the gravitational constant (980 cm/s^2), ρ_s is the mine waste particle density (~2.65 g/cm^3), and ρ_l is the HMP solution density (1.00 g/cm^3). The sedimentation parameter (θ) in $\mu m/min^{0.5}$ is calculated from B and h':

$$\theta = 1000(Bh')^{0.5} \tag{8}$$

The mean particle diameter in μm at each settling time t in minutes is calculated from θ:

$$X = \theta^{-0.5} \tag{9}$$

For a detailed particle size analysis, a summation percentage curve (P versus log X) is plotted using the hydrometer readings from 30 s to 24 h. The % sand, silt, and clay is determined from the curve.

TABLE 13.3

METHOD FOR DETERMINATION OF PARTICLE SIZE DISTRIBUTION (SAND, SILT, AND CLAY) OF MINE WASTE

1 Add 40.0 g of air-dried and sieved (<2 mm) mine waste sample of known residual water content, 250 ml of deionized water, and 100 ml of 50 g/l sodium hexametaphosphate (HMP) solution to a 500-ml wide-mouth plastic bottle
2 Shake for 16 h at 120 cycles/min, quantitatively transfer the suspension to a 1-l sedimentation cylinder by rinsing with deionized water, and make to volume with deionized water. Prepare a reference solution (blank) by adding 100 ml of 50 g/l HMP solution to a 1-l sedimentation cylinder and diluting to volume with deionized water. Stopper and mix the reference solution by inversion several times. Allow the sample suspension and reference solution to come to room temperature
3 At time zero, stopper the sample sedimentation cylinder and mix by inversion several times. Add several drops of amyl alcohol to disperse and foam if necessary
4 For routine clay content determination, take hydrometer readings of the reference solution and sample suspension at 1.5 and 24 h by slowly lowering the hydrometer into the solution and taking the reading at the surface of the liquid after the hydrometer comes to rest. Also record the temperature of the reference solution at 1.5 and 24 h. Keep the cylinders covered with watch glasses between readings. If a more detailed particle size analysis is needed, take hydrometer readings of the sample suspension at 0.5, 1, 3, 10, 30, 60, 90, 120, and 1440 (24 h) min. Also take temperature and hydrometer readings of the reference solution before mixing the sample suspension and at 2 and 24 h
5 Pore the sample suspension through a 53-μm stainless-steel sieve supported over a sink by a ring stand, ring, and large funnel into which the sieve is placed. Rinse the sedimentation cylinder with deionized water to quantitatively transfer all of the sediment onto the sieve. Wash the sand collected on the sieve thoroughly with deionized water to wash through all the silt and clay
6 Tilt the sieve and rinse with deionized water to collect all the sand at one end. Quantitatively transfer the sand with rinsing through a powder funnel into a weighed 150-ml glass beaker
7 Dry the sand overnight at 105°C and weigh. If further fractionation of the sand is required, sieve the sand through nested 1000, 500, 250, 125, and 53-μm stainless-steel sieves using a vibrating sieve shaker for 2 min at speed 8. Weigh each sand fraction collected on the sieves and the residual silt + clay in the receiving pan
8 Calculate the % sand, silt, and clay contents using Eqs. (4)–(13)

For routine clay determination only 1.5 h and 24 h hydrometer readings are needed. A parameter m (slope of summation percentage vs. mean particle diameter at 1.5 and 24 h) is calculated:

$$m = \frac{P_{1.5} - P_{24}}{\ln\frac{X_{1.5}}{X_{24}}} \tag{10}$$

where $P_{1.5}$ is the summation percentage at 1.5 h, P_{24} is the summation percentage at 24 h, $X_{1.5}$ is the mean particle diameter in suspension at 1.5 h (μm), and X_{24} is the mean particle diameter in suspension at 24 h (μm). The % clay is given by

$$P_{2\mu m} = m\ln\frac{2}{X_{24}} + P_{24} \tag{11}$$

Each size fraction of sand after dry sieving is given by

$$\%\text{sand} = \frac{\text{oven-dry weight of sand (g)}}{\text{initial oven-dry weight of sample (g)}} \times 100 \tag{12}$$

The % silt is calculated by mass balance:

$$\%\text{silt} = 100 - (\%\text{sand} + \%\text{clay}) \tag{13}$$

13.4.3 Water retention

The relationship between soil water content and soil water potential is referred to as the water retention or water release characteristic of soil. This characteristic is a fundamental property of soil hydraulics that describes the interaction among soil water and soil particle size distribution, structure, and other components (organic matter, salts, etc.). Most commonly this characteristic is measured with a pressure plate apparatus over a range of soil matric potentials and water contents [19]. Water content is usually expressed either on a dry weight basis or volumetrically, and soil matric potential is inferred indirectly as equivalent to an imposed positive pressure (matric potential is the energy status of water due to adsorption forces on the surfaces of soil particles and by interfacial tension forces within capillaries). This relationship has been used variously to describe the so-called soil constants of 'field capacity' and 'permanent wilting point', which are the water contents at -0.033 and -1.5 MPa, respectively [20,21].

The water retention characteristic is also of interest for understanding the interactions between plants and soils, and how soil water retention affects water movement, availability, and internal plant water relations. The thermocouple psychrometer has recently been used to describe the quantitative characteristics of soil water retention, especially in relationship with plant physiological characteristics [22]. Unlike the pressure plate apparatus, thermocouple psychrometers are sensitive to total water potential (matric plus osmotic forces), are typically sensitive to a much broader range of water potentials than that for which the pressure plate apparatus can safely be designed, and hence can be used to describe the relationship between total water potential and water content over the entire range of plant physiological activity. Table 13.4 compares the relative advantages and disadvantages of each instrument system. Although developed primarily for measurement of water retention in soils, both methods are also applicable to mine wastes.

13.4.3.1 Pressure plate method

The most common method of determining the water retention characteristics of soils is with the pressure plate apparatus [19]. This instrument is used to make indirect measurements of soil matric potential by imposing a known pressure potential on saturated soil samples until free water no longer flows from the system. At this point the assumption is made that the internal pressure potential (ψ_p, the product of the pressure difference between the external atmosphere and the internal chamber pressure and the partial specific volume of water) on the soil sample is equal to the matric potential (ψ_m) of the soil:

$$\psi_p = \psi_m \tag{14}$$

TABLE 13.4

ADVANTAGES AND DISADVANTAGES OF PRESSURE PLATE AND THERMOCOUPLE PSYCHROM-
ETER METHODS FOR MEASURING WATER POTENTIALS

Method	Advantages	Disadvantages
Pressure plate	Does not require elaborate electronic instrumentation	Requires very high pressures (safety issues)
	Does not require precise temperature control	Narrow range of matric potential (0 to -1.5 MPa)
	Highly sensitive over entire pressure range	Matric potential inferred indirectly
		Requires long equilibration times (days)
		Not easily portable
Thermocouple psychrometer	Broad range of sensitivity (-0.15 to -8 or more MPa)	Requires frequent recalibration
	High precision and accuracy possible	Requires isothermal conditions
	Short equilibration times (h)	Insensitive between 0 and -0.15 MPa
	Portable	Requires elaborate electronic instrumentation
	Small sample sizes	Requires frequent cleaning
		Affected by electrical noise

Commercial pressure plate apparatuses are quite adequate to achieve reproducible results if careful procedures are followed. Normally these instruments consist of a heavily constructed steel chamber capable of withstanding pressures in excess of 15 atmospheres, an accurately controlled pressure regulator system, a source of pressurized air or gas with hoses or tubing connected to the chamber, a series of porous ceramic plates with different bubbling pressure capacities, and an outlet for water transport from the samples and chamber. The saturated porous ceramic plates used must be specially constructed to prevent passage of air through the ceramic at specific positive pressures. In addition, the soil samples must be thoroughly saturated with deionized water so that all air spaces and capillaries are free of air. For best results, the system temperature should be controlled within $\pm1°C$ during the period when positive pressure is imposed on the samples.

Mine waste samples may be disturbed or undisturbed cores, and may be sieved (usually <2 mm) beforehand to remove the influences of large rocks. Mine waste samples are saturated from the bottom in standing water in order to allow trapped air to escape and to minimize slaking [19]. This can be achieved by placing 3–5 replicate air-dry samples in shallow open-end rings or other retainers that allow water to infiltrate under the sidewalls and into the mine waste. We recommend that samples be allowed to reach saturation in place for 12–24 h on the surface of a ceramic plate rated for a matric tension ranging from -0.1 to -1.5 MPa that is also saturated with standing water. This procedure negates the need to transfer samples, hence reduces sample loss, and optimizes surface contact between the mine waste and ceramic plate during saturation.

Following complete saturation, excess water is drained from the ceramic plate, and it

and the saturated samples are placed in the pressure chamber apparatus and securely sealed. Pressure is slowly imposed on the system until the desired pressure potential is achieved as indicated by a direct-reading calibrated pressure gauge. Water transport from the mine waste samples through the porous ceramic plate and via the outlet tube in the chamber is visually monitored over time until all water movement ceases. At this point it is assumed that the internal pressure potential and the mine waste matric potential are equal. Two to three days is usually sufficient to achieve consistent and repeatable equilibrium in all textural classes of soils [19]. We assume that mine wastes behave similarly. However, others have allowed more than 30 days to achieve equilibrium [22]. During the equilibrium period, the internal chamber pressure must be carefully monitored and corrected for air loss and pressure drift.

When equilibrium is assumed to be complete, the internal chamber pressure is relieved slowly before opening the system and removing the samples. Water content can be determined on either a volume basis or by weight [19]. To compute water content on a weight basis, the samples are weighed carefully immediately after removal from the chamber, oven dried at 105°C until constant weight is achieved, and then reweighed. Percent water by weight is equal to θ in Eq. (1) $\times$ 100.

13.4.3.2 Thermocouple psychrometer method

Thermocouple psychrometry offers an alternative to the pressure plate method for measuring the water retention characteristics of soils, and for describing the interactive effects of soils and plants over a broad range of total water potential and water content [22]. Two basic types of thermocouple psychrometers are available: (1) the Peltier psychrometer with a total water potential range of sensitivity between about -0.15 to -8.0 MPa, and (2) the Richards-style psychrometer with a range of sensitivity between about -0.15 to -150 MPa [23,24]. Although numerous designs and styles of each type are available, all psychrometers have highly specific requirements and limitations affecting instrument precision and accuracy. Among these, the more important include: (1) calibration, (2) temperature control and thermal stability, (3) cleanliness, (4) precision of electronic instrumentation, and (5) consistency of technique [23,24].

Basically, thermocouple psychrometers are sealed within a small chamber with the sample at constant temperature and allowed to reach vapor pressure equilibrium (usually within 2–4 h). Either using the Peltier effect [24] or by dipping a 'Richards' style [23] thermocouple into water and then rotating it over the sample, the water on the thermocouple is allowed to evaporate into the atmosphere surrounding the sample. At vapor pressure equilibrium, the water potential is related to the relative vapor pressure at constant temperature and pressure by the Kelvin equation:

$$\psi = \frac{RT}{V_\mathrm{w}} \ln a_\mathrm{w} \tag{15}$$

where ψ is the total water potential (MPa), R is the universal gas constant, T is the Kelvin temperature, V_w is the partial molal volume of water, and a_w is the activity coefficient of water at ambient vapor pressure (relative vapor pressure).

For determinations of mine waste water retention, sieved (<2 mm) oven-dry (105°C) mine waste samples of known weight are allowed to saturate in water over night in small

pre-weighed dishes or other containers. The samples are then allowed to dry-down to pre-determined water contents. It is essential that dry-down cycles be used rather than wet-up cycles to minimize the effects of hysteresis [19], and to more precisely approximate the environmental conditions experienced by plants in drying soils. Small mine waste samples of between 5 and 10 g oven-dry weight are preferable to larger samples in order to promote more uniform water contents throughout the sample. At the appropriate water content, each mine waste sample is immediately transferred to and sealed in a chamber containing a pre-calibrated thermocouple psychrometer. The thermocouple psychrometer-chamber assembly is allowed to achieve both vapor pressure and temperature equilibration (usually from 2–4 h in a waterbath at 25°C with Peltier psychrometers such as screen-caged models from either J.R.D. Merrill or Wescor, Inc., Logan, UT, USA, and somewhat shorter times with 'Richards' style instruments, such as the SC-10 from Decagon Devices, Pullman, WA, USA in a controlled temperature room). Water potential is inferred from microvolt measurements of each sample based on calibration [25]. We have found that from 15 to 25 samples for each soil type is sufficient to define the entire relationship between water content and total water potential for most soils over the range of 0– − 8.0 MPa. More paired data sets should be collected near the wet-end of the relationship than the dry-end to more precisely define the correspondence between the two variables.

13.5 CHEMICAL PROPERTIES

Mine waste often contains low levels of major nutrients (e.g., N, P, K) essential for plant growth and potentially toxic levels of metals (e.g., Ni, Cu, Zn, Al, Pb) and metalloids (e.g., As, Se). Some mine wastes have a low pH because of metal sulfide oxidation and still others may contain high levels of soluble salts. Data on the chemical properties of mine waste that may limit plant growth are needed to select appropriate remediation treatments to overcome these limiting factors. In this section, some of the methods of measuring important chemical properties of mine waste are presented.

13.5.1 Salinity and sodicity

Salinity refers to the presence of excessive concentrations of inorganic soluble salts in the mine waste. Excessive levels of soluble salts interfere with plant growth by increasing its osmotic potential (i.e., the work the plant must do to take up water from the mine waste) and forcing the plant to make a physiological adjustment to the presence of the salts [26]. In addition, some of these inorganic salts are toxic to plants at high enough concentrations (e.g., H_3BO_3). Salinity is quantified by measuring the electrical conductivity (EC) of an aqueous extract of the mine waste or the EC of the bulk mine waste in situ [27,28].

Sodicity refers to excessive levels of Na in the mine waste. Sodicity is usually quantified by the exchangeable sodium percentage (ESP) or the sodium adsorption ratio (SAR) [27]. The quantities ESP and SAR are closely related to each other, but SAR is normally easier to measure. Sodicity problems in mine wastes are actually related to the interaction of salinity and sodicity. At SAR levels <10 and EC levels <0.5 dS/m, dispersion of clay minerals occurs resulting in a decrease in permeability of the mine waste. At SAR levels >15, swelling of smectite clays may occur, also decreasing permeability. The relationship

between SAR and EC of infiltrating water needed to maintain permeability that has been used for soils is also useful for mine wastes [27].

Salinity and sodicity problems in mine waste are generally found in arid or semiarid regions where annual evapotranspiration greatly exceeds annual precipitation and are often related to landscape position [26]. Successful reclamation of salt-affected (saline and/or sodic) mine wastes usually involves a combination of mine waste amendments to maintain permeability (e.g., gypsum, mulches), irrigation management, and salt-tolerant plant species [26,29].

Because salinity is usually highly variable spatially on the landscape, the use of field methods to measure bulk EC in situ of mine waste in arid regions where salt-affected soils are widespread can be advantageous [27,28]. For routine characterization, measurement of the EC of an aqueous extract of the mine waste is more common and that is the method given here.

There are a variety of methods available to extract the native aqueous solution in mine waste, both in the field and in the laboratory. These methods include suction lysimeters in the field and displacement, centrifugation, and pressure filtration methods in the laboratory [28]. Because the water content of mine waste varies so widely, use of these solution extraction methods is not always convenient or even possible. More commonly, a saturation extract technique or other ratio of mine waste to water ratio is used to obtain an aqueous solution for EC measurement. Because the saturation extract method is most commonly used for arid region soils and mine wastes, it will be described here. The saturation extract is analyzed for both EC and SAR to assess salinity and sodicity hazards.

If only an appraisal of salinity is needed, then an alternative method is to measure the EC of a saturated paste of the mine waste instead of just the saturation extract [27,28]. This can be done in the field or the laboratory. The saturation percentage (water content at saturation) of the mine waste can also be determined. The saturation percentage is an important physical property of the mine waste because it provides information on water holding capacity in lieu of a complete water retention curve [30]. The EC of a saturation extract can be related to the EC of the saturated paste using the saturation percentage [27,28].

To measure the EC and SAR of a saturation extract of mine waste, a saturated paste of the mine waste is prepared and vacuum filtered (Table 13.5) [28]. The EC of the collected extract is measured using a conductivity meter and electrode. The concentrations of Na, Mg, and Ca in the extract are measured using atomic absorption spectrophotometry (AAS) or inductively coupled plasma–atomic emission spectrophotometry (ICP–AES). The saturation percentage (SP) is given by

$$SP = \frac{\text{weight of added + initial water (g)}}{\text{oven-dry weight of mine waste (g)}} \times 100 \tag{16}$$

The SAR is given by

$$SAR = \frac{Na}{\sqrt{\dfrac{Ca + Mg}{2}}} \tag{17}$$

where Na, Ca, and Mg are the concentrations of these ions in the saturation extract in meq/l (mmol$_c$/l).

TABLE 13.5

METHOD FOR PREPARATION OF A SATURATION EXTRACT OF MINE WASTE

1	Add 200 g of air-dried and sieved (<2 mm) mine waste sample of known water content to a weighed wide-mouth plastic jar with screw-cap lid and weigh jar plus mine waste
2	Add deionized water to the mine waste with stirring until it is near saturation. Cap the jar and allow the mixture to stand for several hours so that the mine waste will equilibrate with the added water. Add more water with stirring to achieve a uniformly saturated mine waste-water paste. A saturated paste glistens as it reflects light, flows slightly when the jar is tipped, slides freely and cleanly off a smooth spatula, and consolidates easily by tapping the jar after a trench is formed in the paste with a spatula [28]. After mixing, allow the paste to stand for 2 h and recheck for saturation. Free water should not collect on the surface, the paste should not stiffen markedly, or lose its glisten. Add an additional known weight of mine waste if the paste is too wet. Once saturation has been attained, re-weigh the jar and contents. Calculate the saturation percentage using Eq. (16)
3	Transfer the saturated paste to Whatman 50 or equivalent filter paper in a Buchner funnel fitted to a vacuum manifold. Apply a vacuum and collect the filtrate for analysis. Measure the EC of the saturation extract using a conductivity electrode and meter. Determine Na, Mg, and Ca in the saturation extract by AAS or ICP–AES and calculate SAR by Eq. (17). The saturation extract may also be analyzed for other solutes of interest (e.g., metals, As, Se) if needed

13.5.2 pH

The pH of mine waste is one of the most useful indicators of mine waste quality for supporting plant growth. It indicates the presence of acidity or alkalinity in the mine waste sample. Mine waste with a pH below 4 probably contains H_2SO_4 as a product of the oxidation of metal sulfides [31]. In the pH range of 4 to 5.5, hydrolysis of exchangeable Al is probably the dominant source of acidity in mine waste as it is in soils [32]. Mine waste in the pH range of about 7.6 to 8.5 likely contains free $CaCO_3$ [32]. A pH near 9 and above indicates the presence of Na and/or K bicarbonate and carbonate minerals [32].

Although a low pH indicates an acidic mine waste, it does not provide any information on the amount of acidity present or on the amount of lime needed to neutralize the acidity. These topics are better discussed in Section 5.5. on sulfur forms and acid–base accounting since they are interdependent.

By far the most common method of measuring mine waste pH is to measure the pH of a 1:1 mine waste/deionized water mixture with a combination pH electrode and meter, and that is the method presented here (Table 13.6) [32]. Other ratios of mine waste to water (e.g., 1:2) are also frequently used [32]. Mine waste pH is also measured in solutions other than deionized water such as 0.01 M $CaCl_2$ or 1 M KCl [32].

13.5.3 Carbon, nitrogen, phosphorus, exchangeable cations, and cation exchange capacity

Usually, mine waste needs supplemental additions of nutrients to support plants and an analysis for plant available amounts will provide information on how much supplemental fertilization is required for reclamation.

Carbon in mine waste typically exists in two forms: organic C and carbonates. Free

TABLE 13.6

METHOD FOR DETERMINATION OF THE pH OF A 1:1 MINE WASTE/DEIONIZED WATER SUSPENSION

1	Add 20.0 g of air-dried and sieved ($<$2 mm) mine waste sample and 20.0 ml of deionized water to a 50-ml disposable plastic beaker
2	Mix the suspension thoroughly with a stirring rod and allow to sit for 2 h
3	Calibrate the pH meter and combination pH electrode with two buffers covering the expected pH range of the mine waste sample
4	Insert the combination pH electrode into the suspension, mix by swirling, and record the pH when the pH drift reaches a minimum

carbonates are typically only found in mine wastes from arid regions, but their presence does influence the availability of other nutrients such as P and some trace elements (e.g., Mn, Fe, Cu, Zn). Many mine wastes, especially subsurface geologic material, contain little organic matter. Only soil O and A horizons that are stripped off as overburden in a surface mining operation would be expected to contain significant amounts of organic C. Topsoil should be stockpiled for use in surface reclamation because they often have higher levels of plant available forms of nutrients than subsurface geologic material. Furthermore, surface soil horizons are an important source of beneficial soil microbial activity that can accelerate the reclamation process.

Thus, an analysis of mine waste for organic matter or organic carbon can indicate the need for added organic matter substrate to re-establish soil microbial activity during reclamation. Organic matter mineralization is also a potentially important source of N and P needed to re-establish nutrient cycling in the mine waste [33]. After reclamation treatments have ceased including any organic matter additions and re-fertilization, native plant communities will need to rely on nutrient cycling to sustain the community. Analysis of reclaimed mine waste for organic matter or organic carbon over time can be used to document soil development on the reclaimed site.

Organic matter content is typically determined by loss on ignition (LOI). There are numerous variations of the LOI method [34]. Most versions differ primarily in ignition times and temperatures. We recommend combustion of an oven-dried sample for 16 h at 450°C (Table 13.7). The LOI is given by

$$LOI\ (\%) = \frac{oven\text{-}dried\ sample\ weight\ (g) - ignited\ sample\ weight\ (g)}{oven\text{-}dried\ sample\ weight\ (g)} \times 100 \qquad (18)$$

There are three main methods for determining total organic C in mine waste: wet combustion, dry combustion, and dichromate oxidation with heating [34]. The wet and dry combustion methods measure total C. In soils without carbonates, all three methods give essentially equivalent results, because all the C is in organic form.

In the wet combustion method, a mine waste sample is digested in a mixture of $K_2SO_4 + H_2SO_4 + H_3PO_4$, the released CO_2 is adsorbed by a CO_2 adsorbing agent (e.g., ascarite), and the adsorbed CO_2 is measured gravimetrically [34]. For mine waste with carbonates, the method can be modified so that only CO_2 from carbonates is released [34]. To determine total organic C (TOC), total carbon is determined on one subsample and carbonates are determined on another replicate subsample. The TOC content is given

by the difference between total C and carbonate C. This method is time consuming and not practical for routine use.

In the dry combustion method, total C in mine waste is determined by measuring the CO_2 released from combusting the sample in a medium-temperature resistance furnace or a high-temperature induction furnace [34]. Automated instruments are available commercially to measure total C in soils [34]. If the mine waste contains carbonates, total C can be measured by dry combustion, carbonate content can be determined by a number of different methods [35], and TOC is calculated as total C − carbonate C.

In the dichromate oxidation with heating method, a sample is digested in H_2SO_4 plus a standard $K_2Cr_2O_7$ solution in a block digester (Table 13.7). The organic C in the sample is oxidized by the $Cr_2O_7^{2-}$ with concomitant reduction of Cr^{6+} to Cr^{3+}. The Cr^{3+} is measured by titration with standardized $Fe(NH_4)_2(SO_4)_2$. Total organic C is given by

$$\%TOC = \frac{AN(0.003)(100)}{W} \tag{19}$$

TABLE 13.7

METHODS FOR DETERMINING ORGANIC MATTER (OM) IN MINE WASTES BY LOSS ON IGNITION (LOI) AND TOTAL ORGANIC CARBON (TOC) IN MINE WASTES BY DICHROMATE OXIDATION WITH HEATING

Organic matter by loss on ignition

1 Prepare a 10-ml porcelain crucible by igniting it for 1 h in a muffle oven at 450°C, cooling it in a desiccator, and weighing it to the nearest 0.1 mg
2 Fill the crucible about half-full with air-dried and sieved (<2 mm) mine waste sample and weigh to the nearest 0.1 mg.
3 Dry the sample for 16 h in a forced-air oven at 105°C, cool in a desiccator, and weigh to the nearest 0.1 mg
4 Ignite the sample in a muffle oven for 16 h at 450°C, cool in a desiccator, and weigh to the nearest 0.1 mg
5 Calculate the water content in the air-dried sample using Eq. (1). The LOI (OM content) is given by Eq. (18)

Total organic carbon by dichromate oxidation with heating

1 Add 10.00 ml of 0.5 N $K_2Cr_2O_7$ and 15.0 ml of concentrated H_2SO_4 to 0.500 g of oven-dried and ground (<0.125 mm) mine waste sample in a block digestor tube, 0.500 g of pure quartz sand in a block digestor tube to serve as a digested blank, and a 250-ml flask to serve as an undigested blank for standardizing the 0.2 N $Fe(NH_4)_2(SO_4)_2$ titrant
2 Heat the tubes in a block digestor to 200°C, digest for 30 min at 200°C, remove the tubes from the digestor, and allow to cool
3 Quantitatively transfer the contents of each tube to a 250-ml flask by thorough rinsing with deionized water and dilute the contents in each flask to about 100 ml
4 Add a stir bar and five drops of o-phenanthroline indicator solution to each flask.
5 Titrate the undigested and digested blanks and sample to the endpoint with 0.2 N $Fe(NH_4)_2(SO_4)_2$. The color changes from yellow to green to blue-green to blue-gray to reddish brown at the endpoint
6 Calculate the TOC content using Eqs. (19) and (20)

$$A = (V_{db} - V_{sample}) \frac{V_{ub} - V_{db}}{V_{ub}} + (V_{db} - V_{sample}) \qquad (20)$$

where A is a correction factor (ml), V_{ub} is the volume of titrant used on the undigested blank to standardize the titrant (ml), V_{db} is the volume of titrant used on the digested blank (ml), V_{sample} is the volume of titrant used on the sample (ml), N is the normality of the $Fe(NH_4)_2(SO_4)_2$ titrant, and W is the sample weight (g).

If the instrumentation is available we recommend using the automated dry combustion method, particularly if the sample contains no carbonates. If the automated dry combustion instrumentation is not available or for samples known to contain carbonates, we recommend using dichromate oxidation with heating provided the Cr-contaminated waste can be disposed of without harming the environment. We do not recommend using the classic Walkley-Black method (dichromate oxidation with no external heating) because of the incomplete oxidation of TOC inherent in the method. Regardless of method, to improve combustion or digestion efficiency and to improve precision because relatively small sample amounts are used, subsamples should be ground to pass a 0.125-mm sieve prior to weighing for analysis.

For most mine wastes, nutrient cycling (N mineralization) cannot be relied on, at least initially, to provide the N needs of the plant community. The initial N needs of the developing plant community must be supplied by N fertilizer or N mineralization from organic matter amendments. Nitrogen fertilizer amendments to mine waste for reclamation are almost always based on the N needs of the plant communities to be established rather than on analysis of the mine waste. Total N is a poor indicator of plant available N in any geologic material including soil. Because of the many forms of N in soil and mine waste and all the transformations among these forms, routine analysis of mine waste for total N and the principal inorganic forms that are available to plants (NH_4^+ and NO_3^-) is seldom worthwhile. The chief advantage of such analyses is that they are a research tool to study N cycling in re-established plant communities and soil development in the reclaimed mine waste.

If an N analysis of the mine waste is needed, we recommend either Kjeldahl digestion or an automated dry combustion method for total N [36]. For NH_4^+ and NO_3^-, we recommend extraction of the mine waste with 2 M KCl followed by analysis of the extract for NH_4^+ by the automated phenate method and for NO_3^- by the automated Cd reduction method [37].

Mine wastes generally contain some amounts of plant available forms of P, K, and other nutrient elements so that an analysis of the mine waste for these plant available forms is worthwhile to determine if and how much supplemental fertilization is needed. There are two common types of methods for extraction of plant available P in soils. For calcareous soils, buffered alkaline extraction methods are commonly used, and for acid soils, dilute acid fluoride extraction methods are recommended [38]. The most common buffered alkaline extractant is pH 8.5, 0.5 M $NaHCO_3$ (Olsen method). The most common dilute acid fluoride extractant is 0.03 M NH_4F + 0.025 M HCl (Bray 1 method). Other methods used for plant available P include extraction with deionized water, 0.05 M HCl + 0.0125 M H_2SO_4 (Mehlich 1 or double acid method), 0.03 M NH_4F + 0.1 M HCl (Bray 2 method), anion exchange resins, and iron oxide-impregnated filter paper strips [38,39]. In addition to these, there are some multi-element extractants that are now commonly used

including pH 7.6, 1 M NH_4HCO_3 + 0.005 M DTPA and 0.25 M NH_4NO_3 + 0.015 M NH_4F + 0.2 M acetic acid + 0.013 M HNO_3 (Mehlich 3 method) [38,39].

Alkaline extractants such as $NaHCO_3$ desorb P associated with the surfaces of Fe and Al oxides and other minerals, but tend not to dissolve Ca phosphate minerals [39]. Alkaline extractants also solubilize some organic matter and associated P. Analysis of the alkaline extract by the ascorbic acid colorimetric method gives inorganic P desorbed from mineral surfaces [38]. Digestion of the extract (persulfate oxidation) followed by colorimetric analysis or direct analysis of the extract by ICP–AES gives total P [38]. The difference between total P and inorganic P gives organic P extracted by the $NaHCO_3$.

Dilute acid fluoride extractants solubilize P associated with Fe and Al oxides [39]. This method should not be used for alkaline mine wastes containing carbonates. The carbonates in the mine waste will neutralize the acid in the extractant. During the neutralization reaction between the carbonates and the acid in the extractant, some Ca phosphate minerals may be dissolved. Also, the NH_4F reacts with $CaCO_3$ to form CaF_2 which leads to formation of secondary precipitates with the solubilized P [38]. Calcium phosphate minerals and carbonates are more likely to be present in mine wastes from arid regions.

For routine analysis of plant available P in mine wastes we recommend the Olsen method for alkaline or calcareous mine wastes and the Bray 1 method for acidic mine wastes (Table 13.8). If information on the forms of P in the mine waste (e.g., organic P, P associated with Fe and Al oxides, P associated with Ca minerals) is needed, then P fractionation methods that have been developed for soils should be used [38].

The exchangeable K content of soils is usually used as a measure of plant available K [40,41]. This would apply to mine wastes as well. Extraction with pH 7, 1 M NH_4 acetate is the most commonly used method to determine exchangeable K in soils and mine wastes [40,41]. The Olsen method for available P in alkaline mine wastes can also be used to determine exchangeable K in these types of mine wastes. The NH_4^+ ion has about the same ionic radius as the K^+ ion, so it gives the most complete replacement of exchangeable K associated with negatively charged sites on layer silicates [41]. The pH 7, 1 M NH_4 acetate method can also be used to extract exchangeable Na, Mg, and Ca in acidic mine wastes. This method should not be used to determine exchangeable Ca in mine wastes containing carbonates because the pH 7, 1 M NH_4 acetate will dissolve a portion of the carbonates and give erroneously high values for exchangeable Ca [42].

The pH 7, 1 M NH_4 acetate method is an example of a buffered salt extraction for exchangeable K and other cations. Unbuffered salts (e.g., NH_4Cl, NH_4NO_3, $BaCl_2$, etc.) can also be used to extract exchangeable cations from mine wastes and offer several distinct advantages over buffered salts [42–44]. The main advantage is that the pH during extraction by unbuffered salts is controlled primarily by the mine waste, whereas during extraction by buffered salts, the pH is controlled by the extracting solution. Thus, unbuffered salts can be used for both acidic and alkaline mine wastes.

For acidic mine wastes, unbuffered salts can extract exchangeable Al in addition to the exchangeable base cations Na, K, Mg, Ca. If pH 7, 1 M NH_4 acetate is used to extract exchangeable bases, then a separate extraction (e.g., 1 M KCl) is needed for exchangeable Al [45]. The sum of the exchangeable base cations plus exchangeable Al will give a suitable estimate of the effective cation exchange capacity (CEC) of the mine waste.

For mine wastes containing carbonates, unbuffered salts tend to dissolve less carbonates, than pH 7, 1 M NH_4 acetate. If an unbuffered salt is used to extract exchangeable

TABLE 13.8

METHODS FOR EXTRACTION OF AVAILABLE PHOSPHORUS IN MINE WASTES

Acidic mine wastes – extraction with 0.03 M NH$_4$F + 0.025 M HCl

1 Add 7.0 ml of 0.03 M NH$_4$F + 0.025 M HCl to 1.00 g of air-dried and sieved (<2 mm) mine waste sample in a 16 × 125-mm capped plastic tube
2 Shake for 1 min at 180 cycles/min
3 Filter through quantitative filter paper and save extract for analysis. Determine P in the extract by the ascorbic acid colorimetric method [38]

Alkaline mine wastes – extraction with pH 8.5, 0.5 M NaHCO$_3$

1 Prepare pH 8.5, 1 M NaHCO$_3$ by dissolving 42.0 g of NaHCO$_3$ in 900 ml of deionized water, adjusting to pH 8.5 with 1 M NaOH, and diluting to 1 l with deionized water
2 Add 40.0 ml of pH 8.5, 1 M NaHCO$_3$ to 2.00 g of air-dried and sieved (<2 mm) mine waste sample in a 125-ml flask
3 Shake for 30 min on an orbital shaker at 150 cycles/min
4 Filter through quantitative filter paper and save extract for analysis. Determine P in the extract by the ascorbic acid colorimetric method [38]

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cations from mine wastes containing carbonates, the extract solution can be analyzed for alkalinity and the exchangeable Ca level corrected for Ca associated with the extracted alkalinity [42]. This will give a more accurate measure of exchangeable Ca and CEC when the exchangeable cations are summed. Similarly, if the mine waste contains gypsum, the unbuffered salt extract can be analyzed for SO$_4$ and the exchangeable Ca corrected for Ca associated with extracted SO$_4$ [42].

The unbuffered salt method we recommend for all mine wastes is extraction with 1 M NH$_4$Cl at a 1:20 solid/solution ratio (Table 13.9). The method has the following advantages:

1. The concentration of salt and soil/solution ratio are sufficient to obtain near complete replacement of all exchangeable cations on the permanent charge sites of the layer silicates in the mine waste
2. The NH$_4$ ion is the optimal size for replacing exchangeable K and will also replace exchangeable Na, Mg, Ca, Al, and other metal cations such as Ni, Cu, and Zn
3. The pH of the extraction is controlled primarily by the mine waste rather than by the extracting solution
4. The exchangeable cation concentrations can be summed to obtain an estimate of the CEC
5. The method can be used for both acidic and alkaline mine wastes
6. Corrections for increases in exchangeable Ca and CEC as a result of dissolution of carbonates and gypsum are possible [42]

A previous version of this method used a mixture of 0.1 M NH$_4$Cl + 0.1 M BaCl$_2$ to simultaneously extract Na, K, Mg, Ca, and Al from acidic and alkaline soils [43]. Unfortunately, in mine wastes high in SO$_4$, Ba precipitates as BaSO$_4$. Therefore, we modified the method for use in mine wastes containing high concentrations of SO$_4$ by eliminating the BaCl$_2$ and increasing the concentration of NH$_4$Cl and the solution to soil ratio to maintain a near complete replacement of the exchangeable cations.

TABLE 13.9

METHOD FOR EXTRACTION OF METAL CATIONS ASSOCIATED WITH LAYER SILICATES IN MINE WASTES USING AN UNBUFFERED SALT (1 M NH_4Cl)

1	Add 20.0 ml of 1 M NH_4Cl to 1.00 g of air-dried and sieved (<2 mm) mine waste sample in a 50-ml plastic centrifuge tube
2	Vortex mix and shake for 2 h at 120 cycles/min
3	Centrifuge for 10 min at 1500 g
4	Decant extract, filter if necessary, and save extract for analysis. Determine metals in extract by ICP–AES

13.5.4 Metals, arsenic, and selenium

Mine wastes often contain potentially toxic levels of many transition and main group metals such as V, Cr, Mo, Mn, Ni, Cu, Zn, Cd, and Pb. The presence of absence of any of these metals depends on the geology and geochemistry of the mineral deposits from which the mine waste is derived. In addition, mine wastes from arid regions often contain potentially toxic levels of As and Se.

There are three types of methods usually used to determine the forms and amounts of these elements in mine wastes. The first group of methods is used to determine the bioavailable (plant available) amounts of these elements in mine waste. Typically, buffered or unbuffered salt extractants are used to measure the amounts of these elements bound to permanent and to some extent pH-dependent charge sites on organic matter and mineral surfaces in the mine wastes. We recommend extraction with 1 M NH_4Cl (Table 13.9) to determine exchangeable cationic metals as a measure of bioavailable metals in acidic mine wastes. The amount of metals extracted from mine waste with 1 M NH_4Cl varies with the pH of the mine waste. As pH decreases, the level of 1 M NH_4Cl extractable metal increases (Fig. 13.1).

The pH of the mine waste is probably the best single predictor of cationic metal species bioavailability. At low pH levels, transition and main group metal cations tend to exist as the free metal cation species (e.g., Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}) and are more soluble, more mobile, and more bioavailable to plants and other organisms than at higher pH levels [46]. As pH increases, the free metal cations hydrolyze, sorb to mineral and organic matter surfaces, and precipitate as metal hydroxides [46]. The net result of these reactions is to decrease metal cation bioavailability. Extraction with 1 M NH_4Cl is a rapid and simple means of estimating metal cation bioavailability as a function of mine waste pH.

There are many other methods for measuring the amounts of bioavailable metals in mine wastes including extractions with dilute mineral acids (e.g., 0.1 M HCl), chelates (e.g., DTPA), and ion-exchange resins [47–49]. Probably the most popular method for determining bioavailable amounts of metals in soils and mine wastes is extraction with pH 7.3, 0.005 M DTPA (diethylenetriaminepentaacetic acid), although multi-element extractants such as pH 7.6, 1 M NH_4HCO_3 + 0.005 M DTPA and the Mehlich III extractant are also commonly used.

The DTPA method is most applicable to near-neutral and alkaline soils and mine wastes [50]. Because the stability of metal ion–chelates decreases with decreasing pH, the DTPA method should not be used for very acidic mine wastes (pH < 4). It can be used for

moderately acidic mine wastes if the pH of the mine waste is used along with the chelate extractable amounts of metals to interpret the results. Because the DTPA method is so widely used, we present it here (Table 13.10).

Many transition metals and main group elements exist as oxyanion species (e.g., vanadate (VO_4^{3-}), chromate (CrO_4^{2-}), molybdate (MoO_4^-), arsenite (AsO_2^-), arsenate (AsO_4^{3-}), selenite (SeO_3^{2-}), and selenate (SeO_4^{2-})). These species tend to sorb to metal (chiefly Fe and Al) oxide surfaces as a function of pH [51]. Unlike metal cations, oxyanion sorption is maximized at acid pH levels and decreases at alkaline pH levels. Oxyanion species sorbed to metal oxide surfaces can be a readily available source of these elements for plant uptake.

To measure the amount of oxyanion species sorbed to metal oxide surfaces, we recommend extracting the mine waste with a 0.01 M solution of KH_2PO_4/K_2HPO_4 buffered at pH 7.2 [52]. The method is presented in Table 13.11 and is applicable to acidic and alkaline mine wastes containing vanadate, chromate, molybdate, arsenite, arsenate, selenite, and/or selenate species. The excess concentration of phosphate anions in the extracting solution provides a near complete replacement of the other oxyanions sorbed to Al and Fe oxide surfaces. Phosphate anions tend to be more strongly sorbed to the metal oxide surfaces than other oxyanions. The main inorganic species of As (arsenite and arsenate) and Se
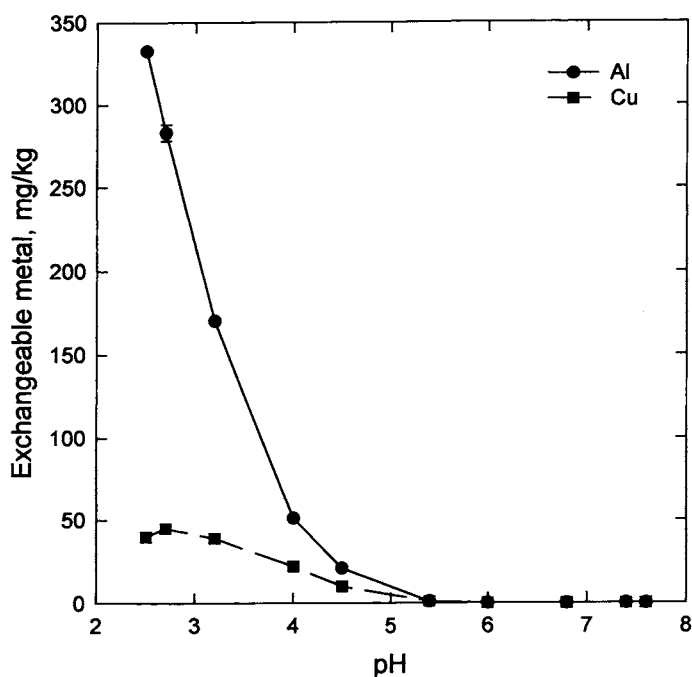
Fig. 13.1. Relationship between NH_4Cl extractable Al (●) and Cu (■) and pH for mine waste from the McLaren Mine, New World Mining District, MT, USA. The mine waste was limed with $Ca(OH)_2$ to various pH levels and wet and dried three times to allow the lime to react with the mine waste prior to extraction with NH_4Cl.

TABLE 13.10

METHOD FOR EXTRACTION OF METALS FROM NEAR-NEUTRAL AND ALKALINE MINE WASTES
USING pH 7.3, 0.005 M DTPA

1	Prepare the DTPA extraction solution by dissolving 1.96 g of DTPA (diethylenetriaminepentaacetic acid), 14.92 g of TEA (triethanolamine), and 1.47 g of $CaCl_2 2H_2O$ in 900 ml of deionized water, adjusting to pH 7.3 ± 0.05 using 6 M HCl, and diluting to 1 l with deionized water
2	Add 20 ml of DTPA extraction solution to 10 g of air-dried and sieved (<2 mm) mine waste sample in a 125-ml flask
3	Shake for 2 h on an orbital shaker at 150 cycles/min
4	Filter through quantitative filter paper and save extract for analysis. Determine metals in extract by ICP–AES

(selenite and selenate) in the phosphate extracts can be measured using hydride–AAS or hydride–ICP–AES techniques [53–55].

Element solubility in the mine waste can also be assessed by analyzing a saturation extract (Table 13.5) for the element species of interest using graphite furnace AAS [56], ICP–mass spectrometry [57], or in the case of As and Se, hydride–AAS or hydride–ICP–AES [53].

The second group of methods is used to determine the amounts of transition and main group metals and nonmetals associated with specific organic matter and mineral phases. These methods selectively dissolve specific solid phases and the elements associated with them [48,58–61]. They can be used sequentially or individually, although some of the extractants, when used individually, will dissolve more than one type of solid phase.

Considerable controversy has developed over the accuracy of selective extraction methods and much of the controversy is still largely unresolved [61–67]. There are two principal disadvantages of sequential extraction methods. The first is that the extraction reagents are not completely selective for the target phase. The second is that elements released during one extraction may be re-adsorbed by the remaining solid phases in the mine waste residue.

Despite these limitations we have found that selective and sequential extractions can

TABLE 13.11

METHOD FOR EXTRACTION OF OXYANIONS FROM MINE WASTE USING pH 7.2, 0.01 M $KH_2PO_4/$ K_2HPO_4

1	Add 20.0 ml of pH 7.2, 0.01 M KH_2PO_4/K_2HPO_4 to 2.00 g of air-dried and sieved (<2 mm) mine waste sample in a 50-ml plastic centrifuge tube
2	Vortex mix and shake for 2 h at 120 cycles/min
3	Centrifuge for 10 min at 1500 g
4	Decant extract, filter if necessary, and save extract for analysis. Determine inorganic As and Se species in the extract by hydride AAS or hydride ICP–AES [54,55]. Determine Cr(VI) by the s-diphenylcarbazide colorimetric method [52]. Determine V by the gallic acid colorimetric method [91]. Oxyanion metals in the phosphate extract can also be determined by concentrating the metals with a chelation-extraction method using APDC (ammonium pyrrolidine dithiocarbamate) followed by analysis of the metals by ICP–AES [48]

give reliable information on the chemical associations of trace elements in mine waste, particularly when augmented by a mineralogical analysis using x-ray diffraction and other solid phase characterization methods. Selective extraction methods offer the best means of evaluating chemical associations of trace elements with poorly crystalline phases that are not amenable to study by other methods [68].

The methods presented here describe sequential extractions of metals associated with cation exchange sites on layer silicates, carbonates, Mn oxides, noncrystalline Fe oxides, crystalline Fe oxides, and organic matter + sulfides (Table 13.12) and are modifications of popular methods in the literature [59,69–73]. If the mine waste does not contain carbonates, then the carbonate extraction step should be omitted.

Solid and liquid phases are separated by centrifugation and extracts are filtered through glass fiber syringe filters. The residue from each extraction is washed with ethanol to remove entrained solution and allowed to air-dry overnight before proceeding with the

TABLE 13.12

METHODS FOR SEQUENTIAL EXTRACTION OF METALS ASSOCIATED WITH LAYER SILICATES, CARBONATES, MANGANESE OXIDES, IRON OXIDES, AND ORGANIC MATTER + SULFIDES IN MINE WASTES

A. *Exchangeable metal cations associated with layer silicates – extraction with 1 M NH_4Cl*
1   Add 10.0 ml of 1 M NH_4Cl to 0.500 g of air-dried and sieved (<2 mm) mine waste sample in a 50-ml plastic centrifuge tube, vortex mix, and shake for 2 h at 120 cycles/min
2   Centrifuge for 10 min at 1500 *g*, decant extract, filter if necessary, and save extract for analysis. Determine metals in extract by ICP–AES.
3   Wash residue three times with 5 ml of 95% ethanol by vortex mixing, centrifuging for 5 min at 1500 *g*, and discarding ethanol washes

B. *Metals associated with carbonates – extraction with pH 5, 1 M NH_4 acetate*
1   Add 25.0 ml of pH 5, 1 M NH_4 acetate to the air-dried residue from step A and let sit for 0.5 to 1 h to allow initial reaction with carbonates to subside. Vortex mix and shake for 24 h at 120 cycles/min
2   Centrifuge for 10 min at 1500 *g*, decant extract, filter if necessary, and save extract for analysis. Determine metals in extract by ICP–AES
3   Wash residue with 95% ethanol as in step A

C. *Metals associated with Mn oxides – extraction with pH 2, 0.1 M NH_2OHHCl*
1   Add 25.0 ml of pH 2, 0.1 M NH_2OHHCl to the air-dried residue from step B, vortex mix, and shake for 30 min at 120 cycles/min
2   Centrifuge for 10 min at 1500 *g*, decant extract, filter if necessary, and save extract for analysis. Determine metals in extract by ICP–AES
3   Wash residue with 95% ethanol as in step A

D. *Metals associated with noncrystalline Fe oxides – extraction with 0.2 M NH_4 oxalate + 0.2 M oxalic acid in the dark*
1   Add 25.0 ml of 0.2 M NH_4 oxalate + 0.2 M oxalic acid to the air-dried residue from step C, vortex mix, cover the shaker with a black plastic bag, and shake for 2 h at 120 cycles/min
2   Centrifuge for 10 min at 1500 *g*, decant extract, filter if necessary, and save extract for analysis. Determine metals and S in extract by ICP–AES
3   Wash residue with 95% ethanol as in step A

TABLE 13.12 (*continued*)

A. Exchangeable metal cations associated with layer silicates – extraction with 1 M NH₄Cl

E. Metals associated with crystalline Fe oxides – extraction with 0.2 M NH₄ oxalate + 0.2 M oxalic acid + 0.1 M ascorbic acid in a boiling-water bath
1   Add 10 ml of 0.3 M NH₄ oxalate + 0.3 M oxalic acid and 5 ml of 0.3 M ascorbic acid to the air-dried residue from step D, vortex mix, place in a boiling water bath for 15 min, remove, and allow to cool for 15 min
2   Centrifuge for 5 min at 1500 g, decant extract into a 50-ml volumetric flask, and repeat extraction (step 1), centrifuging, and decanting steps, adding the second extract to the volumetric flask
3   Resuspend residue in 5 ml of oxalate solution, centrifuge, and decant into the volumetric flask. Dilute extract to volume with deionized water, mix, and save for analysis. Determine metals and S in extract by ICP–AES

F. Metals associated with organic matter and sulfides – digestion in aqua regia and 30% H₂O₂
1   Add 5 ml of 95% ethanol to the residue from step E, vortex mix, and quantitatively transfer the residue to a 125-ml conical beaker by rinsing with small volumes of ethanol. Evaporate to dryness in an oven at 80 °C.
2   Add 1 ml of concentrated HNO₃ and 3 ml of concentrated HCl to the oven-dried residue. Digest on a hot plate at low heat until nearly dry. Repeat digestion in HNO₃ and HCl twice more
3   Add 1 ml of concentrated HNO₃ and 4 ml of 30% H₂O₂ and digest over low heat to near dryness. Repeat digestion in HNO₃ and H₂O₂ twice more
4   Add 1 ml of concentrated HCl, warm the beaker on a hot plate, and rinse down the sides with 1% HCl. Quantitatively transfer the digest and residue from the conical beaker to a 50-ml volumetric flask by rinsing with small volumes of 1% HCl. Dilute digest to volume with deionized water, mix, and save for analysis. Determine metals and S in digest by ICP–AES

next extraction. Washing with ethanol instead of water produces a cleaner phase separation during centrifugation. Water washes tend to disperse the residue resulting a potentially serious loss of solid phase from one step to the next. Air-drying is sufficient to remove the ethanol entrained in the residue so it does not interfere with the next extraction. The extracts are analyzed for the elements of interest by AAS or ICP–AES.

The third group of methods is used to determine the 'total recoverable' amounts of the elements of interest in the mine waste. Most of these methods involve digestion with strong acids in open beakers on a hot plate (e.g., USEPA method 3050) [74], tubes in a block digestor [48], or closed containers in a microwave oven (e.g., USEPA method 3051) [75]. Although USEPA method 3050 is useful for determining anthropogenic sources of metals in soils and sediments, it can give incomplete element recoveries for some mine wastes because of incomplete solid phase dissolution. The microwave method is to be preferred over digestion with open beakers on a hot plate because less time is required for digestion, there is less likelihood for contamination because the digestion is done in a sealed container, more complete dissolution of the solid phase can be obtained because of a higher pressure during digestion, and sample loss and volatilization can be minimized because of the sealed container [76].

Two methods for total recoverable elements in mine wastes are presented here: diges-

TABLE 13.13

METHODS FOR TOTAL RECOVERABLE METALS IN MINE WASTES

Digestion in HNO_3 + $HClO_4$ with a block digestor

1   Prepare a digestion mixture of 1400 ml of reagent grade concentrated HNO_3 plus 400 ml of reagent grade concentrated $HClO_4$
2   Add 20 ml of digestion mixture to 0.500 g of oven-dried and ground (<0.125 mm) sample in a digestion tube
3   Digest at 200 °C in a block digestor until white fumes of $HClO_4$ are produced. (Alternatively, digest sample on a hot plate in a 125-ml conical beaker with a small funnel in the mouth of the beaker to promote refluxing). For safety, carry out the digestion in a $HClO_4$ fume hood with wash-down capability
4   Allow sample to cool, transfer with rinsing to a 25-ml volumetric flask, fill to volume with deionized water, mix, and save for analysis
5   Determine concentrations of elements of interest using AAS, ICP–AES, or ICP-MS

Lithium metaborate fusion

1   Thoroughly mix 0.200 g of oven-dried and ground (<0.125 mm) sample with 0.800 g of lithium metaborate fusion flux
2   Transfer the mixture to a graphite crucible and heat at 1050°C in a high-temperature furnace for 10 min. For safety, use long-handled tongs, heat-protective gloves, lab-coat, and face shield to load and unload the crucible in the furnace
3   Carefully pour the melt into a wide-mouth jar containing 100 ml of 4% HNO_3
4   Shake the jar until the flux dissolves completely
5   Determine concentrations of elements of interest by AAS, ICP–AES, or ICP-MS

tion in HNO_3 + $HClO_4$ with a block digestor [48] and lithium metaborate fusion [77] (Table 13.13). For reasons of safety, the HNO_3 + $HClO_4$ digestion must be carried out in a $HClO_4$ fume hood with wash down capability. If a block digestor is not available, the digestion can be done with conical beakers on a hot plate. A small funnel placed in the mouth of each conical beaker will promote a reflux action essential for efficient digestion but still allow for slow evaporation of the HNO_3. The lithium metaborate fusion method uses a relatively small sample size and may not be suitable for all elements and mine wastes because of potential volatilization at the high temperature used for fusion (1050°C).

13.5.5 Lime requirement, sulfur forms, and acid–base accounting

Many mine wastes, particularly coal and metal mine wastes, contain metal sulfide minerals. Pyrite (FeS_2) is the most common of the metal sulfides and is widely distributed [31]. When exposed to water and oxygen, pyrite and other metal sulfides are oxidized. Metal oxides (e.g., hydrous ferric oxide) and H_2SO_4 are the products of the redox reactions of H_2O and O_2 with metal sulfides. The term acid rock drainage (ARD) refers to H_2SO_4-contaminated drainage waters from regional near-surface deposits of metal sulfides. The term acid mine drainage (AMD) refers to H_2SO_4-contaminated drainage waters from mine workings (pits, tunnels, etc.) in geologic deposits containing metal sulfides.

The mechanisms and factors influencing the rates of metal sulfide oxidation are known primarily from studies of pyrite oxidation [78–81]. Ferric iron and oxygen are the principal oxidants:

$$FeS_2(s) + 3.5O_2(aq) + H_2O = Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad (R1)$$

$$FeS_2(s) + 14Fe^{3+} + 8H_2O = 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \qquad (R2)$$
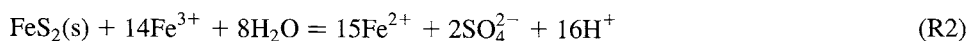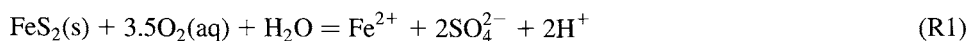
Ferric iron (Fe^{3+}) oxidizes pyrite much faster than O_2 does, but the process is thought to begin with oxidation by dissolved O_2.

Ferrous iron (Fe^{2+}) produced during pyrite oxidation can itself be oxidized by O_2 to Fe^{3+}. The Fe^{3+} produced from the oxidation of Fe^{2+} by O_2 can also oxidize pyrite according to R2. The ensuing auto-oxidation cycle generates considerable amounts of acidity (16 moles of H^+ for every mole of FeS_2). The oxidation rate of Fe^{2+} by O_2 is slow and is independent of pH below pH 3. Above pH 3, the oxidation rate increases with increasing pH.

Certain microorganisms such as *Thiobacillus ferroxidans* catalyze the oxidation of Fe^{2+} by O_2, greatly increasing the oxidation rate. Under acidic conditions, microbially catalyzed oxidation of Fe^{2+} is more important than abiotic oxidation. Under neutral or alkaline conditions, abiotic oxidation becomes significant. Temperature and pH also affect the oxidation of metal sulfides by their influence on the growth of *T. ferroxidans*.

The large quantities of acidity generated during the oxidation of metal sulfides in mine waste present special problems for restoration of native plant communities. The pH of acidic native soils is not less than 4, but the acidity produced by metal sulfide oxidation can drive the pH of mine waste down to values in the 2–3 range [31]. This excessive level of acidity will promote the dissolution of many primary and secondary minerals [82]. Metal cation solubility and mobility greatly increase at low pH leading to phytotoxicity [83]. Furthermore, low pH levels have a deleterious effect on root uptake of water and nutrients and soil microbial activity [83].

Although much effort has been expended on developing methods to inhibit metal sulfide oxidation with varying degrees of success [31], the usual approach taken in mine waste remediation is to add lime to neutralize the excess acidity. Therefore, it is necessary to have accurate methods for measuring the amount of acidity already produced in mine waste where some metal sulfide oxidation has occurred and for predicting the potential amounts of acidity that could be generated from unoxidized metal sulfides still in the mine waste. Mine waste from inactive and abandoned mines that has been subjected to physical and chemical weathering with time are often already acidic because the amounts of acidity generated exceeded whatever capacity for neutralization the mine waste may have initially had. Unweathered mine waste will have mostly potential acidity. Even alkaline mine wastes from arid regions and elsewhere may contain sufficient quantities of metal sulfides to generate enough acidity to neutralize the alkalinity in the waste rock and may eventually become acidic.

There are many methods available to measure existing and potential acidity in mine waste [84]. Existing acidity (EA) can be measured by a lime incubation method or by a soil test lime requirement buffer method [85,86]. Potential acidity (PA), also called acid potential (AP), is usually estimated by measuring the forms of S in the mine waste [84]. If the acid neutralizing potential (ANP) of the mine waste is also measured, then the difference between ANP and AP can be used to determine if the mine waste has sufficient alkalinity to neutralize the potential acidity or a deficit of alkalinity and must be amended with additional liming material. This is the basis for the acid–base accounting

(ABA) method for assessing whether unweathered mine waste may become acidic in time and how much additional liming material is needed to neutralize potential acidity as it is generated.

Despite its widespread use, the ABA method has two principal shortcomings which can lead to unreliable results. First, the method can only measure the potential amounts of acidity to be neutralized in the case of ANP or produced in the case of AP. The method does not measure the actual rates of acid neutralization or production that will occur under field conditions. Second, the methods used to measure ANP and AP frequently under or over estimate these quantities. Sulfur fractionation methods are used to distinguish acid-forming sulfide minerals from nonacid-forming sulfate minerals to obtain a more accurate estimate of AP [87]. When the mineralogy of the mine waste is dominated by iron sulfides, then Fe in the extracts can also be used to compute the AP [84]. Unfortunately, many metal sulfates and metal sulfides are incompletely dissolved by the reagents used to extract the S forms [87]. These measurement errors result in either over-liming of the mine waste or under-liming which leads to reclamation failure.

One option for estimating potential acidity is to bypass the measurement of S or Fe in extraction methods that attempt to distinguish between acid and nonacid forming S minerals and to titrate the acidity released when the metal sulfide is oxidized by a strong oxidant such as H_2O_2 [84]. Unfortunately, this method is unreliable for mine wastes with complex mineralogy, although a modified version of the original method has been found to work reliably for lignite overburden samples [88].

Because the ABA method only measures potential acidity or potential acid neutralization, a kinetic method was developed in an attempt to measure the actual amounts of acidity produced during simulated chemical weathering of the mine waste in a humidity cell [89]. Although this is a more dynamic approach to predicting the acid generating potential of the mine waste, the lengthy time required to obtain data by this method negates its use for routine determinations. Despite the shortcomings of the ABA method, it is still the only rapid and routine method for estimating AP and ANP and is widely used.

If the mine waste is strongly acid (pH < 4), significant amounts of acidity have already been produced during metal sulfide oxidation and there is little need for measuring ANP. The total lime requirement of the mine waste will be the amount of lime needed to neutralize the existing acidity measured by lime incubation or buffer methods plus the amount of lime needed to neutralize any remaining potential acidity as it is generated. If the mine waste is only weakly acidic, near-neutral, or alkaline, the metal sulfides may be unoxidized or only slightly oxidized, and ANP should be measured. The lime requirement of the mine waste will be equal to the difference between ANP and AP.

The lime incubation method for existing acidity is the standard against which all buffer methods are compared. Although requiring much more time to complete than a buffer method, it is the most accurate method for measuring soil or mine waste acidity. Many soil test buffer methods have been developed for measuring lime requirement [85,86]. The SMP buffer method is perhaps the most widely used. Although, it was developed to measure the lime requirement of acid soils in which exchangeable Al is the most dominant form of acidity, it can also be used for mine wastes which have H_2SO_4 as the major source of acidity, provided the capacity of the buffer is not exceeded. To ensure that the capacity of the SMP buffer is not exceeded, a wider buffer/solid phase ratio may be needed with mine wastes than with acid soils.

A lime incubation method using saturated Ca(OH)$_2$ solution and the SMP buffer method are presented here (Table 13.14). An example of the relationship between lime rate and mine waste pH is shown in Fig. 13.2. Lime requirement in terms of pure CaCO$_3$ to raise the pH to a given target pH can be calculated from the regression equation for the curve. The relationship between lime rate and pH typically flattens out at the equilibrium pH for CaCO$_3$ when all of the acidity has been neutralized. A 1-week incubation should be sufficient for neutralizing existing acidity from H$^+$ ions and hydrolysis of metal cations. Additional acidity produced after this time is probably from metal sulfide oxidation and should be measured by the ABA method.

For the SMP buffer method, the amount of lime (megagrams or metric tons of pure CaCO$_3$ per hectare of mine waste to a 15-cm depth) needed to increase the pH of mine waste to 7.0 is given by

$$LR = 85.23 - 12.29 BpH \qquad (21)$$

where LR is lime requirement in Mg/ha to a 15-cm depth and BpH is the measured pH of the SMP buffer/mine waste suspension. This equation was derived from tabulated data in the literature [85]. The SMP buffer method is capable of providing lime requirements

TABLE 13.14

SMP BUFFER AND LIME INCUBATION METHODS FOR DETERMINING LIME REQUIREMENTS FOR NEUTRALIZING EXISTING ACIDITY IN MINE WASTES

SMP buffer method

1　The SMP buffer consists of 1.8 g/l p-nitrophenol, 3.0 g/l K$_2$CrO$_4$, 53.1 g/l CaCl$_2$2H$_2$O, 2.0 g/l Ca acetate, and 2.5 g/l triethanolamine (TEA) adjusted to pH 7.5 with NaOH or HCl. Details for preparing large volumes of this buffer are available [86]

2　Shake 10 g of air-dried and sieved (<2 mm) mine waste with 20 ml of SMP buffer for 15 min and let the suspension stand for 15 min

3　Measure the pH of the buffer - mine waste suspension using a combination pH electrode and meter and calculate the lime requirement from the buffer pH using Eq. (21)

Lime incubation method

1　Prepare a saturated Ca(OH)$_2$ solution by suspending 1.85 g of Ca(OH)$_2$ in 1 l of deionized water and shaking for 48 h

2　Determine the pH of the mine waste (Table 13.6). If the pH is > 5.5, lime is probably not needed. If the pH is between 4.0 and 5.5, add 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 ml of saturated Ca(OH)$_2$ solution and 48, 46, 44, 42, 40, 38, 36, 34, 32, and 30 ml of deionized water to 2.5-g samples of air-dried and sieved (<2 mm) mine waste in a series of 250-ml flasks. If the pH of the mine waste is < 4.0, add 4, 8, 12, 16, 20, 24, 28, 32, 36, and 40 ml of saturated Ca(OH)$_2$ solution and 46, 42, 38, 34, 30, 26, 22, 18, 14, and 10 ml of deionized water to 2.5-g samples of prepared mine waste. Each ml of saturated Ca(OH)$_2$ solution added to 2.5-g sample is equivalent to a lime rate of 1 t CaCO$_3$/kt mine waste (1 t/a (6-inch depth)

3　Insert foam plugs in the mouths of the flasks and shake the samples at 150 rpm on an orbital shaker for 1 h each day for a 1-week equilibration period

4　Measure the pH of the mine waste suspensions using a combination pH electrode and meter

5　Calculate a regression equation for a graph of lime rate vs. pH. Use the regression equation to calculate the lime rate needed to attain the desired pH (usually 7)
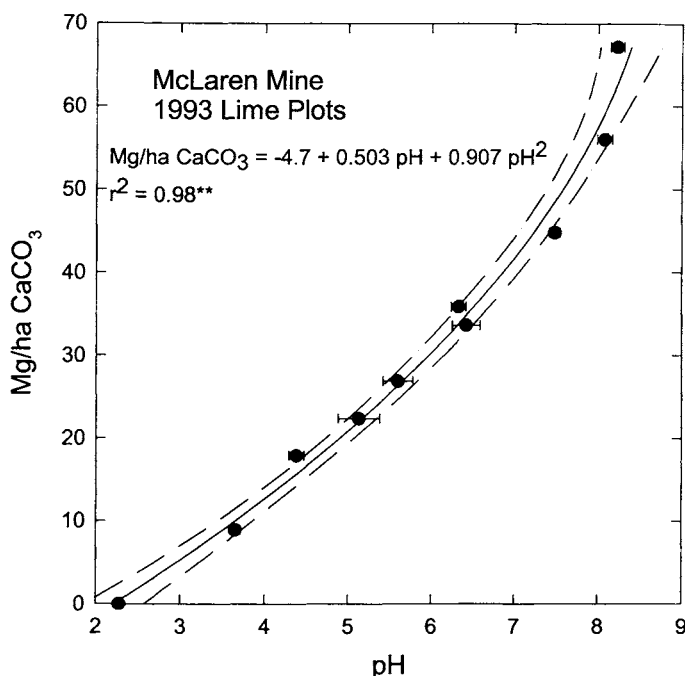
Fig. 13.2. Relationship between lime rate (Mg $CaCO_3$/ha) and pH for a mine waste sample from the McLaren Mine, New World Mining District, MT, USA. The titration curve was constructed by incubating increasing volumes of saturated $Ca(OH)_2$ solution equivalent to increasing rates (Mg/ha) of added $CaCO_3$ with mine waste for 1 week according to the method outlined in Table 13.14.

similar to those determined by lime incubation. Table 13.15 shows calculated lime requirements for neutralizing existing acidity by the lime incubation and SMP buffer methods for several mine waste samples from the Summitville Superfund Site in Colorado. The agreement between the two methods for four of the five samples is very good considering that they use very different procedures. For sample TSSP2-SE, the lime incubation method gave a lime requirement almost double that for the SMP method. There may be some additional acidity in that sample that was neutralized during the lime incubation method that did not react with the SMP buffer.

Methods for measuring ANP and forms of S (and hence AP) in mine waste are summarized in Table 13.16 [87,90]. Acid neutralization potential (ANP) is determined by reacting a sample of mine waste with standardized HCl and measuring the unreacted HCl with standardized NaOH. The ANP of mine waste is given by

$$ANP = 0.1(V - T)N\frac{MW_{CaCO_3}}{2000}\frac{100}{W} \tag{22}$$

where ANP is in tons (t) $CaCO_3$/1000 tons (kt) mine waste, V is the volume of added 1 N HCl (25 ml), T is the volume of 1 N NaOH used in the titration, N is the normality of the titrant (1 N), MW_{CaCO3} is the molecular weight of pure $CaCO_3$ (100 g/mol), W is the sample weight, and the other numbers are conversion factors. The ANP method measures

TABLE 13.15

COMPARISON OF SMP BUFFER METHOD WITH LIME INCUBATION METHOD FOR MEASURING EXISTING ACIDITY (EA) IN MINE WASTE SAMPLES FROM THE SUMMITVILLE SUPERFUND SITE, SUMMITVILLE, CO

Sample	Lime requirement by SMP buffer method (t $CaCO_3$/kt mine waste)	Lime requirement by lime incubation method (t $CaCO_3$/kt mine waste)
TSSP1-S	12	12
TSSP2-SE	18	30
TSSP 3	15	15
BMD 83	14	10
NWDA 55	14	10

the maximum acid neutralization potential of the mine waste from carbonates, silicates, and other minerals. In actuality, not all of the ANP in mine waste will be realized in field situations.

To determine AP, the forms of S are determined by sequentially extracting the mine waste with hot water, 4.91 M HCl, and hot 1.99 M HNO_3 (Table 13.16) [87]. The hot water extraction removes nonacid-producing sulfate minerals (chiefly $CaSO_4$), the HCl extrac-

TABLE 13.16

METHODS FOR MEASURING ACID NEUTRALIZATION POTENTIAL (ANP) AND SULFUR FORMS (ACID POTENTIAL (AP)) IN MINE WASTES

Acid neutralization potential (ANP)

1 Heat 1.00 g of oven-dried and ground (<0.125 mm) mine waste sample in 25 ml of standardized 1 M HCl for 30 min in a boiling water bath. Add 100 ml of deionized water and cool to room temperature
2 Titrate with standardized 1 M NaOH to a phenolphthalein endpoint. Calculate ANP using Eq. (22)

Sulfur forms and acid potential (AP)

1 Add 1.000 g of oven-dried and ground (<0.125 mm) mine waste sample to weighed quantitative filter paper in a filter funnel and leach with 150 ml of boiling deionized water. Retain leachate for analysis, dry residue and filter paper at 40°C, and reweigh
2 Leach residue and filter paper from step 1 with 150 ml of 2:3 (4.91 M) HCl followed by 10 ml of deionized water. Retain leachate for analysis, dry residue and filter paper at 40°C, and reweigh
3 Remove residue from filter paper, weigh, and add to a weighed 250-ml conical flask. Add 150 ml of 1:7 (1.99 M) HNO_3, weigh, cap with a foam plug, and heat for 6 h in a boiling water bath. Cool, reweigh, and filter through weighed quantitative filter paper. Rinse flask with 30 ml of deionized water and pour through filter paper. Retain leachate for analysis, dry residue and filter paper at 40°C, and reweigh
4 Analyze each leachate for S by ICP–AES. Analyze subsample of original sample and final extracted residue for total S using LECO S analyzer if available. Calculate S concentrations and lime requirements to neutralize potential acidity using Eqs. (23)–(30)

tion removes what are considered to be acid-producing sulfate minerals, and the HNO_3 extraction dissolves sulfide minerals [87]. The residual S remaining in the mine waste after the sequential extraction is considered to be organic S in the case of coal mine waste and is assumed to be nonacid-producing [84]. In the case of mine waste from hard-rock mining operations, the residual S is considered to be more inert metal sulfides that could not be extracted in the HNO_3 step and are assumed to be acid-producing [87].

There are basically two ways in which the forms of S in the extracts or residual mine waste can be quantified. Probably the simplest approach is to measure total S in the various extracts by ICP–AES. If a LECO S analyzer is available, then total S is measured in subsamples of the original sample and each of the residues after extraction. The various forms of S are given by

$$\text{NAP SO}_4\text{-S} = \frac{S_{H_2O}\ V}{10\,000\ W} = \text{Total } S_{OS} - \text{Total } S_{H_2O\ \text{residue}} \tag{23}$$

$$\text{AP SO}_4 - S = \frac{S_{HCl}\ V}{10\,000\ W} = \text{Total } S_{H_2O\ \text{residue}} - \text{Total } S_{HCl\ \text{residue}} \tag{24}$$

$$\text{Sulfide-S} = \frac{S_{HNO_3}V}{10\,000W} = \text{Total } S_{HCl\ \text{residue}} - \text{Total } S_{HNO_3\ \text{residue}} \tag{25}$$

$$\text{Residual-S (organic or non-extractable sulfide-S)} = \text{Total } S_{HNO_3\ \text{residue}} \tag{26}$$

where NAP SO_4-S is non-acid-producing SO_4-S (%), AP SO_4-S is acid-producing SO_4-S (%), S_{H_2O} is total S in the hot H_2O extract (mg/l), S_{HCl} is total S in the HCl extract (mg/l), S_{HNO_3} is total S in the HNO_3 extract (mg/l), defaultV is extract volume (ml), defaultW is sample weight (g), total S_{OS} is the total S in the original sample (%), total $S_{H_2O\ \text{residue}}$ is total S in the residue after the hot H_2O extraction (%), total $S_{HCl\ \text{residue}}$ is total S in the residue after the HCl extraction, and total $S_{HNO_3\text{residue}}$ is total S in the residue after the HNO_3 extraction.

The lime requirements to neutralize the potential acidity from each S fraction are given by

$$\text{LR}_{\text{AP SO}_4\text{-S}} = 23.44\text{AP SO}_4\text{-S} \tag{27}$$

$$\text{LR}_{\text{sulfide-S}} = 31.25 \text{ sulfide-S} \tag{28}$$

where LRAP SO_4-S is lime requirement to neutralize potential acidity from acid-producing sulfate minerals (e.g., jarosite) (t $CaCO_3$/kt mine waste), $LR_{\text{sulfide-S}}$ is lime requirement to neutralize potential acidity from sulfide minerals (t $CaCO_3$/kt mine waste), AP SO_4-S is the acid-producing SO_4-S content of the mine waste (%), and sulfide-S is the sulfide S content of the mine waste (%). The factor 23.44 is the amount (tons) of $CaCO_3$ needed to neutralize the acidity produced by 1000 tons of mine waste containing 1% AP SO_4-S and is based on the stoichiometric neutralization of the amount of acidity produced from the mineral jarosite $(KFe_3(SO_4)_2(OH)_6)$ as the representative acid-producing sulfate mineral. The factor 31.25 is the amount (tons) of $CaCO_3$ needed to neutralize the acidity produced by 1000 tons of mine waste containing 1% of sulfide-S. It is based on the stoichiometric reaction of 2 moles of $CaCO_3$ with 4 moles of H^+ produced from 1 mole of FeS_2 (pyrite).

If the residual S in the mine waste sample is known to be organic S (e.g., coal), then

assume that no potential acidity will be generated by the residual S. If however, the mine waste is from a hard-rock metal mine, then assume the residual S is unreacted sulfide-S and use Eq. (28) to calculate the lime requirement to neutralize potential acidity based on the residual S value.

The total lime requirement needed to neutralize all sources of acidity (existing + potential) is given by

$$LR_{total} = LR_{existing} + LR_{AP\ SO_4-S} + LR_{sulfide-S} + LR_{residual-S} \tag{29}$$

where $LR_{existing}$ is the lime requirement of existing acidity as measured by lime incubation or buffer methods, the other lime requirements are defined as before, and all LR values are expressed in units of t $CaCO_3$/kt mine waste.

If the mine waste contains no existing acidity and has acid neutralization potential (ANP), then the acid–base account (ABA) is given by

$$ABA = ANP - [LR_{AP\ SO_4-S} + LR_{sulfide-S} + LR_{residual-S}] \tag{30}$$

where all quantities are in t $CaCO_3$/kt mine waste.

Some simplification in lime requirement calculations is possible depending on the forms of S and sources of acidity present in the mine waste. If unoxidized pyrite or closely related minerals are the only metal sulfides present in the mine waste, then the lime requirement needed to neutralize potential acidity can be based on a total S analysis of the mine waste and calculated from Eq. (28) assuming total S = sulfide-S. If some oxidation has occurred, but the only source of existing acidity is from H_2SO_4 and the only source of remaining potential acidity is from unoxidized pyrite or closely related metal sulfides, then lime requirement can also be based on a total S analysis.

A comparison of lime requirement based on total S with lime requirement calculated from measuring existing acidity with the SMP buffer method and potential acidity based on measurement of sulfide-S showed reasonable correspondence between the methods for mine wastes from the Summitville Superfund Site (Fig. 13.3). For many of the samples, lime requirement based on total S was less than lime requirement based on existing and potential acidities. The simplified method assumes that all existing acidity is from H_2SO_4 derived from oxidation of pyrite. However, other reactions (primarily Al hydrolysis) also contribute to existing acidity. Ignoring acidity from other sources besides oxidation of pyrite is often less of an error than the sampling error caused by spatial variability of existing and potential acidity found at many sites.

The rather large spatial variability typically found at most reclamation sites presents special challenges for remediation treatments. If lime additions are based solely on the mean lime requirement, however determined, for a given site, then many areas are likely to be under-limed. Over-liming, although wasteful, is not as serious a problem as under-liming. Under-liming in the worst case scenario can lead to reclamation failure and attendant environmental damage if acidity and metals migrate off-site.

Plotting lime requirements on a site map, stratifying the area to be reclaimed into subareas based on similar lime requirements, and using a statistical approach to calculating the amounts of lime that need to be applied will help avoid under-liming problems. This is the approach being used at the Summitville Superfund Site. The 300-ha Summitville site was subdivided using a grid system where each block is equal to 0.4 ha. Each 0.4-ha subarea is represented by a composite mine waste sample consisting of four equal
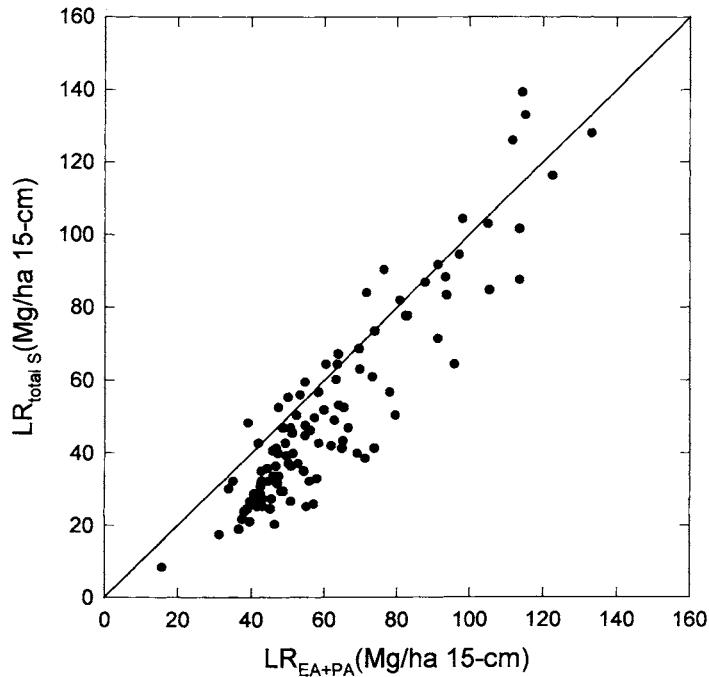
Fig. 13.3. Relationship between lime requirement calculated from total S data and lime requirement calculated from buffer pH (existing acidity (EA)) and sulfide-S (potential acidity (PA)) data for Summitville, CO mine waste samples. The total S, SMP buffer pH, and sulfide-S data are from a report on Summitville Superfund Site mine waste sample analysis [12]. The lime requirements are given by $LR_{total\ S}$, Mg/ha(15-cm depth) = (31.25)(2.24) total S, % and LR_{EA+PA}, Mg/ha(15-cm depth) = (85.23 − 12.29BpH) + (31.25)(2.24) sulfide-S, % where BpH is SMP buffer pH, the 31.25 factor converts % total or sulfide-S to t $CaCO_3$/kt mine waste (t/a(6-inch depth), $\rho_b = 1.5$ g/cm^3), and the 2.24 factor converts t/a(6-inch depth) to Mg/ha(15-cm depth).

subsamples each representing 0.1 ha. Each composite sample was analyzed for total S and lime requirements were calculated using the simplified method described above (LR, Mg/ha (15-cm depth) = (31.25)(2.24)(total S, %). The lime requirements were plotted on a map of the site, and the site was divided into subareas based on the physical setting of the site and lime requirements. Mean, standard deviation, and 95% confidence interval (CI) statistics were calculated for each subarea. The amount of lime to be applied to each subarea is equal to the mean plus the 95% CI such that there is only a 5% probability of encountering an underlimed 0.4-ha block in any given subarea following liming.

Acid neutralization potentials, potential acidities, and lime requirements for mine wastes are usually expressed in units of t $CaCO_3$/kt mine waste. However, in the case of large remediation sites, it is often more convenient to express lime requirements in terms of weight of lime per unit area of mine waste surface. The t $CaCO_3$/kt mine waste unit is equivalent to t $CaCO_3$/acre (a) mine waste to a depth of 6 inches for a mine waste with a bulk density of 1.5 g/cm^3. If the mine waste has a different bulk density, then multiply the t $CaCO_3$/kt mine waste by the measured bulk density divided by 1.5. To convert from t/a for a 6-inch depth to Mg/ha for a 15-cm depth, multiply by 2.24.

13.5.6 Metal sulfide weathering

Methods for determining acid potential based on measurement of forms of S in the mine waste can only estimate the maximum potential acidity that the mine waste can generate provided the analysis is complete and accurate. These measurements alone provide no information on rates of oxidation of metal sulfides under field conditions. Incubation of mine waste in humidity cell methods, although they provide important information on rates of metal sulfide oxidation, are too time consuming for routine use.

A method based on selective extraction of the forms of Fe oxides and oxidized and reduced S in mine waste can provide some information on the degree of weathering of metal sulfides under site conditions. The method requires that pyrite be one of the major metal sulfide minerals in the mine waste. The method also relies on the finding that poorly crystalline Fe oxide minerals such as ferrihydrite and schwertmannite tend to be present in the mine waste during active pyrite oxidation because they are some of the first mineral weathering products formed. As the mine waste ages the more reactive metal sulfides are oxidized and the poorly-crystalline Fe oxides are replaced by crystalline Fe oxides such as goethite and hematite [78]. The method also makes use of the findings that sulfide-S predominates in inert mine wastes, unweathered mine wastes, or mine wastes in the very early stages of oxidation; a mixture of sulfates and sulfide-S is found in mine wastes undergoing active chemical weathering; and sulfate-S predominates in aged and weathered mine wastes.

The method uses a portion of the sequential extraction procedures in Table 13.12. A sample of mine waste is sequentially extracted with 0.2 M NH_4 oxalate + 0.2 M oxalic acid in the dark for noncrystalline Fe oxides, 0.2 M NH_4 oxalate + 0.2 M oxalic acid + 0.1 M ascorbic acid in a boiling-water bath for crystalline Fe oxides, and aqua regia (1 part concentrated HNO_3 + 3 parts concentrated HCl) + 30% H_2O_2 on a hot plate for metal sulfides. The two oxalate extracts remove sulfate associated with the noncrystalline and crystalline Fe oxides (oxidized S) and the aqua regia + 30% H_2O_2 digestion dissolves most metal sulfides (reduced S). Total Fe oxide content is given by the sum of noncrystalline plus crystalline Fe oxide contents and total S is given by the sum of oxidized plus reduced S. Mine waste pH measurements and information on the amount of time the mine waste has been exposed to wet oxidizing conditions are useful in helping to interpret the results of the sequential extraction analysis.

Three different types of mine wastes can be identified by this procedure based on the degree of chemical weathering they have been subjected to (Table 13.17). The data in Table 13.17 are from application of the sequential extraction method to mine waste samples from the McLaren Mine, New World Mining District, MT, USA and the Summitville Superfund Site, Summitville, CO, USA and were selected to illustrate the potential utility of this approach to mine waste characterization.

Unoxidized or inert mine wastes have a low ratio of noncrystalline to total Fe oxides, but a low ratio of oxidized S to total S (Table 13.17). They may have slightly acid, near-neutral, or even alkaline pH levels depending on the mineralogical composition of the mine waste. Although, these mine wastes have been exposed to a wet oxidizing environment for prolonged periods of time (decades in the case of some of the mine waste at the McLaren Mine), little of the metal sulfides have oxidized indicating that they have a low potential for generating acidity. These mine wastes may have a coating of secondary

TABLE 13.17

MINE WASTE PROPERTIES INDICATING METAL SULFIDE WEATHERING

Location	pH	Non-crystalline/ total Fe_2O_3	Oxidized/ total S
McLaren Mine, MT			
Unoxidized mine waste	7.1	0.29	0.08
Actively oxidizing mine waste	2.0–4.4	0.78–0.85	0.22–0.34
Oxidized mine waste	2.1–3.4	0.07–0.29	0.68–0.85
Summitville, CO			
Slightly oxidized mine waste (ANP = 48.4 t $CaCO_3$/kt mine waste)	6.9	0.86	0.07
Actively oxidizing mine waste	2.6	0.62	0.52
Oxidized mine waste	2.6	0.33	0.86

mineral weathering products over the pyrite surfaces and are a prime candidate for surface analysis techniques to determine why they remain inert in a wet oxidizing environment. These mine wastes probably do not need liming treatments because even though an ABA may show that they have an AP potential in excess of the ANP, kinetic considerations argue against any immediate treatments.

Actively oxidizing mine wastes are characterized by a high ratio of noncrystalline to total Fe oxides indicating that active metal sulfide oxidation is underway because the Fe oxide mineralogy is dominated by poorly crystalline forms produced early in the weathering process. Actively oxidizing mine wastes will have a low pH if all of the ANP has been used (Table 13.17), but they may have near-neutral or even alkaline pH levels if the mine waste is in the early stages of weathering and still has unreacted ANP (Table 13.17). These mine wastes typically also have a substantial quantity of unreacted metal sulfides that will produce more acidity as oxidation proceeds. If the amount of unoxidized metal sulfide remaining in the mine waste is high, then this type of mine waste is a prime candidate for treatment by microencapsulation [31]. Microencapsulation refers to formation of a rind of secondary mineral weathering products such as ferric phosphate or hydrous ferric oxide around the pyrite grains preventing further oxidation of the pyrite and associated acid production.

Weathered or oxidized mine wastes are characterized by a low pH, a low ratio of noncrystalline to total Fe oxides indicating that there is currently little active metal sulfide oxidation, and a high ratio of oxidized to total S (low ratio of reduced to total S) indicating that most of the metal sulfides have already oxidized (Table 13.17). This type of mine waste needs to be limed to neutralize the acidity produced during oxidation of the metal sulfides, but there is little chance of any subsequent acid generating potential.

There is a similarity between the sequential extraction methods for the forms of S to determine AP (Table 13.16) and the sequential extraction methods for oxidized and reduced S to assess metal sulfide weathering (Table 13.12). The hot water and HCl extractions dissolve sulfate minerals, although incompletely [87], and the oxalate extracts remove SO_4-S associated with Fe oxides. The hot HNO_3 and aqua regia + 30% H_2O_2 extractions were designed to dissolve metal sulfides. However, the hot HNO_3 method is

known to incompletely extract some metal sulfides [87], and the aqua regia + 30% H_2O_2 method has not been evaluated using different metal sulfide minerals to test extraction efficiency. Total S is determined by summing the S content each extract or by measuring S with the LECO S analyzer. As yet, no rigorous comparison between the two sets of methods has been conducted to determine if the sequential extractions for assessing metal sulfide weathering (Table 13.16) can substitute for the sequential extractions for S forms (Table 13.12). If the methods are found to give comparable results, then ABA and metal sulfide weathering can be assessed with a single set of methods.

13.6 CONCLUSIONS

Mine wastes are a complex mixture of primary minerals and secondary mineral weathering products that usually present an inhospitable environment for establishment of native plant communities. Simple and reliable characterization methods are needed to assess the physical and chemical properties that may inhibit revegetation. Information on the properties of mine wastes are also needed to select the most reliable and cost-effective remediation treatments that will lead to successful site reclamation and restoration.

The complex nature of mine wastes presents many unique challenges to the analyst faced with determining the most important physical and chemical properties that influence selection of remediation treatments. The methods presented here represent some of the most widely used methods and were chosen based on method simplicity, reliability, and cost considerations. There still remains a great need to develop more simple, reliable, and cost-effective mine waste characterization methods. In particular, rapid and more accurate methods are needed to assess potential acidity from metal sulfide oxidation.

ACKNOWLEDGEMENTS

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Chapter 14

Certified reference materials for quality control of measurements in environmental monitoring

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CONTENTS

14.1 PARTICULAR ASPECTS OF ENVIRONMENTAL ANALYSIS

The United Nations' Summit on the protection of the environment held in Rio de Janeiro (Brazil) in June 1992 was a confirmation that environmental protection has gained world-wide interest and, is now a priority for several countries. The public concern and, consequently, the economic and political impact of environmental protection, has led to the development of several projects. In some cases regional, national or international regulations (e.g. European Community (EC) Directives) or international conventions

such as the Paris and Oslo convention for the North Sea, or the Barcelona convention for the Mediterranean Sea, require monitoring programmes and sometimes introduce maximum allowed concentrations for certain contaminants. These monitoring programmes have one common prerequisite: that measurements of various parameters are necessary to evaluate the situation and follow its evolution. The results of the determinations performed provide a basis for the decisions taken by the authorities and for possible actions. Their effect is again evaluated using measurements that are conducted over long periods of time. Trends and even the rate of decontamination processes may be established, as a result of which actions will be modified. The economic and legal impact of the decisions (closing of factories, restraints in the workplace, waste management etc.) and the human effect (unemployment or the displacement of populations as in Seveso in Italy or Times Beach in the USA) can be enormous.

At industrial level regulations on safety of products and processes as well as the image of the company in the public have led to drastic evolution in the perception of environmental impact of activities. Industry has slowly included environment in its primary objectives. The adoption of quality management systems based on the newly issued ISO 14000 series of standards [1] as an extension of the ISO 9000 series [2], is a sign of this concern and new approach. Competitiveness is not anymore considered as incompatible with environmental concerns. This new approach implies that the impact on environment is measured in order for it to be kept under control. For industry this means monitoring input and output of raw and manufactured products, refuse and waste, as well as of energy consumption. Accurate measurements will optimise production but also minimise threats to the environment. For all these reasons, measurements for environmental monitoring have to be as reliable as possible.

14.2 RELIABILITY OF DATA

Reliable data are the result of a chain of actions which starts with the proper definition of the problem to be solved. This should lead to a clear identification of the parameter(s) concerned. It includes the target samples to be selected, the sampling strategy, and the proper sampling technique to be applied. When adequate samples have been analysed the results should be reported in such a manner that those who have to draw the conclusions and to take action, can do so with all possible assurance.

Between the sampling and the reporting of the data lies the difficult work of the analyst in the laboratory. This closes the circle of the total quality control procedure as defined by Griepink and Maier [3]. Proper determinations of chemical parameters mean that precise (repeatable and reproducible) and true – meaning accurate – results are delivered. To achieve the best possible precision within the state of the art the analysts have to work in the most favourable environment. The laboratory has to be organised according to a quality assurance program. This includes infrastructure, personnel qualification and motivation, workload, maintenance of apparatus, proper chemicals, adequate management, etc. A comprehensive quality assurance manual should be available. The best quality systems available nowadays for testing laboratories are based on the ISO 25/EN 4500/1–2 standards [4,5].

Only methods validated in detail should be used. These methods should be under

statistical control [3]. The survey of the method can be followed by control charts, as described in Section 3. It has been demonstrated that a good quality assurance programme is essential to the accuracy and precision of analytical results [6]. When all possible measures have been applied in the laboratory to improve the precision, the analyst can concentrate on obtaining trueness, i.e. accuracy.

14.3 THE NEED FOR ACCURACY

The International Organisation for Standardisation (ISO) defines accuracy as: 'the closeness of the agreement between the test result and the accepted reference value' [7].

As said before, an enormous number of analyses are being performed for the purpose of monitoring the environment and to control the environmental impact of industrial activity. Together with the diversity, the number of materials to be determined, and the wide range of concentrations, one has to consider the complexity of the matrices to be studied. Current analytical techniques with powerful instruments, which are often to a large extent auto-mated (including the interpretation of the data), have made it possible to routinely deter-mine concentrations of 10^{-15} g/g or quantities of 10^{-15} g. This achievement in sensitivity has been obtained along with the high throughputs of analyses which are normal nowa-days. However, these very high sensitivities do not necessarily improve accuracy [8]. A good reproducibility over time is not sufficient to allow one to follow trends and demon-strate the effects of actions carried out to improve the quality of the environment. Mini-mising the effects on environment by changing processes in industry also requires accuracy as changes in the tested systems and products are often important. The applica-tion of results to modelling, and the development of eco-toxicological and ecological theory, etc., as well as improvements in equipment and methodology oblige economic players and scientists to handle accurate data. There are still too many analysts who confuse accuracy with precision: the within-laboratory repeatability and reproducibility are both essential for the monitoring of the environment but are not sufficient. Without trueness associated to precision, results from different groups in different parts of the world are not comparable, although they should be.

Accurate measurements are expensive but bad measurements cost much more. An example illustrating the usual accuracy achieved by laboratories of good reputation is given in Fig. 14.1. This example dealt with measurements of air in the workplace. Tenax sorption tubes were charged with vapours (approx. 1.0 mg) of benzene, toluene and *m*-xylene and distributed for analysis to several well-trained and selected laboratories. In the first interlaboratory study the results differed from the target value by more than the legally allowed limit (10%). Only after three exercises were the participants able to meet the required accuracy. Numerous other examples can be given [9–15]. To achieve and prove the required accuracy, several conditions have to be fulfilled.

14.4 HOW TO ACHIEVE ACCURACY

Concern about the lack of reliability of results has grown over the last decade. First attempts to improve traceability of information of analytical data were made at manage-ment and organisational level [3]. Quality systems under which laboratories had to act
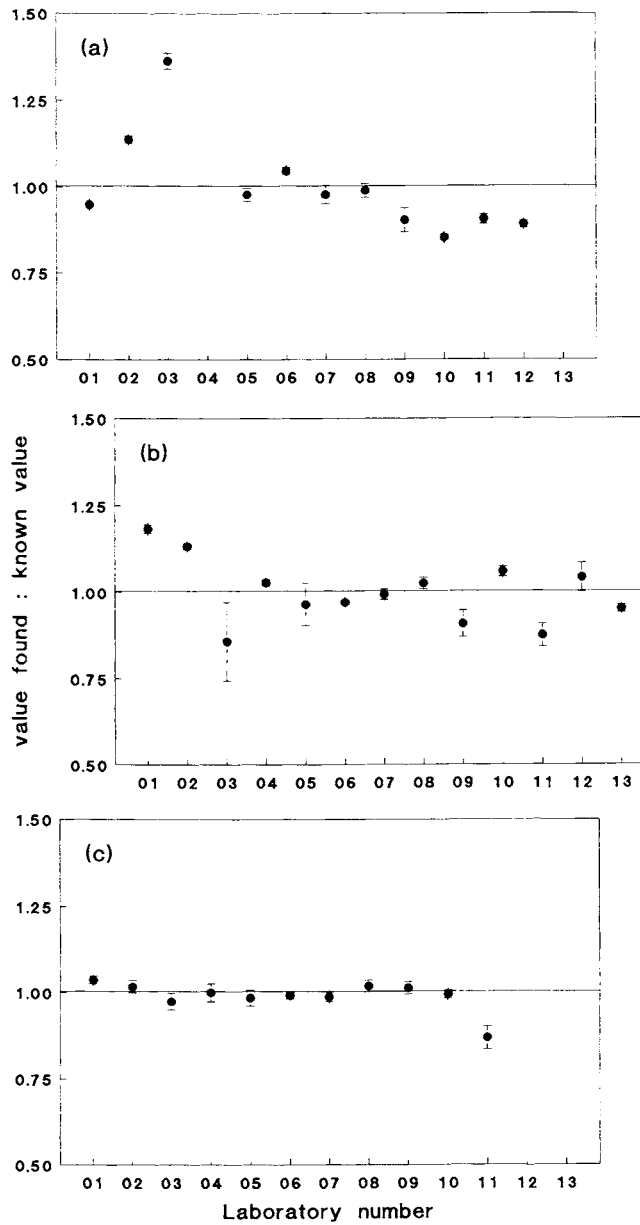
Fig. 14.1. Results of three intercomparisons for toluene trapped on Tenax (mean value ± 1SD). Known value = value calculated from the preparation data of the tubes. (a–c) First, second, third round. After each round the results were examined and discussed in a meeting with all participants [52].

were defined. Good Laboratory Practice (GLP) rules were first established for toxicological testing laboratories by the Food and Drug Administration (FDA) and the Environ-

mental Protection Agency (EPA) in the USA, as well as the Organisation for Economic Co-operation and Development (OECD). For other fields of measurements the quality criteria and requirements are given in the international standards issued by ISO and the European Committee for Standardisation (CEN) e.g. the ISO 25 [4] and EN 45001–2 [5]. These regulations and standards describe the requirements for laboratory organisation and management, competence of management and personnel, the working procedures, e.g. the need for proper calibration of instrumentation, the use of control charts, the participation in external quality assessment schemes (interlaboratory studies), etc. They will soon be replaced by the ISO 17025 standard

These standards and regulations, even if followed strictly, do not guarantee accurate results. To achieve accuracy of measurements technical quality assurance and control tools have to be applied. Accurate results can be achieved and demonstrated in various ways.

14.4.1 Comparison with a different method

Each method has its own sources of error to which those of the analyst applying it have to be added. For spectrometric methods it can be the digestion of the matrix. Although this is not a problem in instrumental neutron activation analysis (INAA), INAA may have errors due to shielding, insufficient separation of gamma-peaks, etc. An independent method, e.g. inductively coupled plasma optical emission spectrometry (ICP-OES) instead of INAA, can be used to verify the results of routine analysis. If both methods are in good agreement it is likely that the results obtained by the routine method will not be affected by a systematic bias. The conclusion is most valid when both methods differ widely. If the methods used for this verification have similarities, such as the pre-treatment, a comparison may overlook systematic errors due to the common step. If the technician is not sufficiently experienced with the comparison method this may even create additional errors.
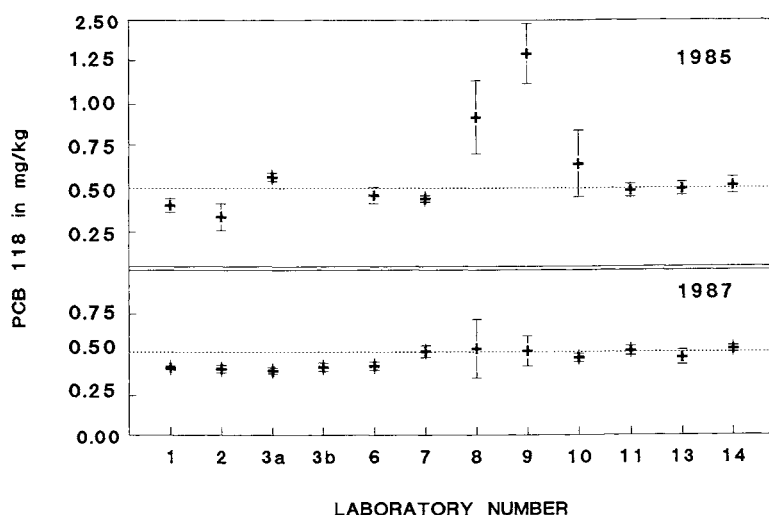
Fig. 14.2. Improvement in the between laboratory agreement for the determination of CB 118 in two similar fish oils: herring in 1985 and mackerel in 1987 [53].

14.4.2 Comparison with other laboratories

The laboratories participating in interlaboratory studies often observe that their results are scattered. Fig. 14.2 illustrates such a situation for the determination of polychlorinated biphenyls (PCBs) in fish oils. Fig. 14.3 shows the achievements of a group of laboratories working on the determination of polychlorodibenzo-*p*-dioxins (PCDDs) and polychloro-
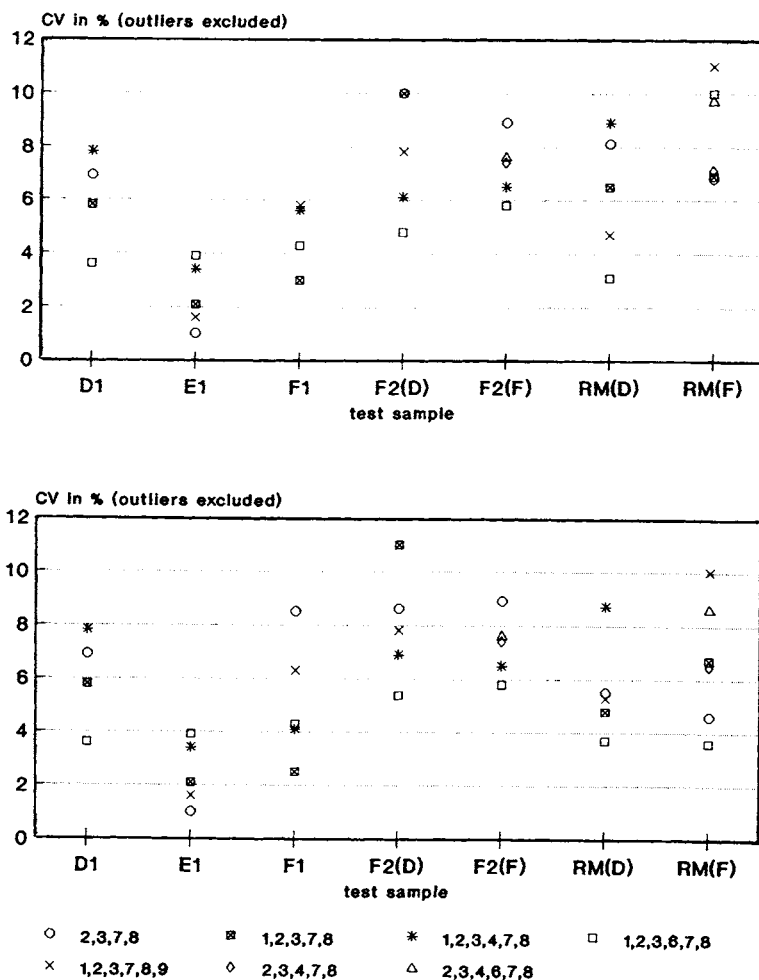
Fig. 14.3. Interlaboratory studies on the determination of PCDDs and PCDFs in fly ash (upper graph for all participants; lower graph for constant participants). The group followed the scheme presented in Fig. 14.4. The data presented in the upper and lower graphs are the coefficient of variation of the mean of means after exclusion of outliers on a technical basis following the discussion between the participants. D1, 1st solution PCDD; E1, 2nd solution PCDD (with properly controlled and common calibrants); F1, PCDD and PCDF in clean fly ash extract; F2(D), PCDDs in raw fly ash extract; F2(F), PCDFs in raw fly ash extract; RM(D), PCDDs in CRM 429; RM(F), PCDFs in CRM 429 (fly ash extract).
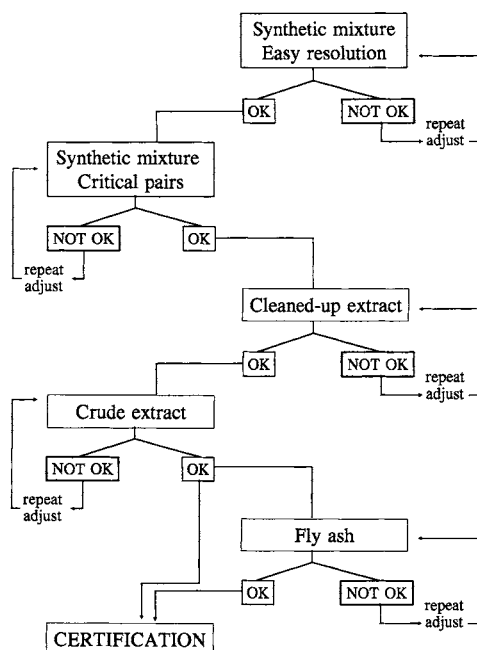
Fig. 14.4. Improvement scheme for the determination of PCDDs and PCDFs in fly ash [54].

dibenzo furans (PCDFs). They used a so-called step-by-step approach consisting of several interlaboratory studies. In each exercise they had to analyse adapted materials to validate each step of their analytical procedure. Fig. 14.4 details the scheme followed in this latter study. The participation in such interlaboratory studies, combined with a critical discussion of the results, is very useful for obtaining a high level of accuracy. Many similar examples have been reported so far [16]. Experience indicates that once a good level has been achieved, continued participation is necessary to maintain the good measurement quality [17]. It can be expected that in the near future the opportunity to participate in interlaboratory studies will increase, as such participation is recommended by accreditation bodies following the prescription of the ISO 25 [4] and EN 45000 series of norms [5]. Several bodies are contemplating the organisation of interlaboratory studies, with or without proficiency objectives. International, as well as national, organisations are normalising the rules for the organisation of such studies in order to guarantee a maximum benefit for the participants [18]. However, there is not always a proper proficiency scheme in the field of interest of the laboratory.

14.4.3 Certified reference materials

Definitions (ISO) [19]:

- *Reference material (RM)*: A material or substance one or more properties of which are sufficiently homogenous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

- *Certified reference material (CRM)*: A reference material accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence.

The use of CRMs is the easiest way to achieve accuracy. Certified reference materials of good suppliers link the user's results to those of the international scientific community. Additionally, they enable the user to verify his performance at any desired moment.

14.5 USE OF CRMs

14.5.1 Calibration and traceability

If one wishes to investigate the accuracy of an analytical procedure it is important to study each step of the process, from weighing the sample to the final determination. No measurement can be valid if all instruments used in the course of the analytical procedure are not properly calibrated (e.g. calibration of balances, volumes of glassware and pipettes, instruments). Modern methods of final determination are based on the measurement of a signal which must be correlated with the concentration of the substance of interest in the unknown sample. This can be a chemical element, a chemical form of an element (e.g. metal speciation) or a chemical compound in organic analysis. The correlation is established by means of a calibration curve. The solutions used to construct the calibration curve must be prepared with chemicals of an appropriate purity and verified identity and stoichiometry [20]. The matrix effects should be estimated and the calibration solutions should be 'matrix matched'. If all steps of the procedure are fully under control and all uncertainties have been estimated the measurements of the analytical sample (digest, solution, clean-extract, etc.) can be considered to be traceable to defined chemical species and consequently to the mole of substance.

In fact, in chemical analysis the measurement process contains steps where the sample is physically destroyed (e.g. acid digestion, fusion, calcination) or the analyte is extracted from the matrix. To ensure accuracy it is necessary to demonstrate that no losses or contamination occurred in the course of the sample treatment. The laboratory can verify the analytical procedure with a reference material having a matrix similar to the unknown sample and certified in a reliable manner. Any disagreement between the certified value and the value determined by the laboratory indicates the presence of an error in the analytical procedure. When there is no disagreement between the found and the certified value the laboratory can conclude that the method has been properly applied with this particular sample. The CRM plays a role similar to a metrology transfer standard and will allow the laboratory to link the measurement process to an internationally recognised reference [21].

Unfortunately, as will be discussed in more detail later the total similarity of CRM and real samples cannot be always assured for reasons of stability or homogeneity. Therefore, some uncertainty remains regarding the accuracy of determination of real samples.

14.5.2 Accuracy of a method

When developing a new analytical method or apparatus and after having evaluated all its critical points the analyst has to prove the accuracy of the measurements. Usually he compares its results with those obtained with a classical method. This implies that this classical method is fully under control in his laboratory. However, some critical factors, such as the influence of laboratory contamination, cannot be solved in that laboratory itself and would need to be investigated in another laboratory. It is in fact much easier to use a CRM to evaluate the accuracy if a suitable CRM exists. CRMs will help to study both components of accuracy: precision and trueness. ISO Guide 33 gives indications to analysts how to use CRM for both purposes [22].

CRMs may also be used to test a standardised method when it is applied for the first time in the laboratory or when a new technician is applying it. The use of a standardised method is not a guarantee of accuracy [23] and does not protect against mistakes and criticism. Sometimes very qualified laboratories have difficulties in putting written standards into practice. These standard procedures are usually elaborated by very senior analysts who may forget that they will be applied by less qualified people in charge of routine measurements. Unfortunately, often due to economical constraints, new or revised standard procedures are not always tested, validated, in interlaboratory studies. Consequently, difficulties in application or understanding, inaccurate translation, may remain. It also has to be stressed here that standard procedures may block the improvement of the methodology applied in the laboratories. In fact, by prescribing a procedure one may prevent the use of recently developed methods or instruments which may be more sensitive and less subject to systematic errors. Unless the standard procedure is essential to define the parameter to be measured (see item d in Section 6), it would be more effective to authorise the use of any method whose accuracy has been proven by the use of a suitable CRM.

14.5.3 Evaluation of working standards and statistical control charts

When a laboratory works at a constant level of high quality, fluctuations in the results are small [24]. As soon as a method is under control in a laboratory, control charts should be made to detect possible drifts. A control chart is a graphical representation of the results obtained for the measurement of a reference sample or working standard. This should be a material similar to the unknown samples, with a proven homogeneity and a good stability. Fig. 14.5 shows examples of control charts. For a more rapid detection of trends (introduction of systematic errors) or drifts, Cusum charts (cumulative sum) can be used [9,25,26].

Working standards also called laboratory reference materials (LRM), may additionally be compared with similar CRMs. This can help to maintain the trueness of the method. It must be noted that this 'traceability to CRMs' is not easy to achieve in one single laboratory and that it would be preferable to have the LRM analysed by different good laboratories, applying different methods, as is often done by certification bodies. In Cusum charts, the need of a reference value – X_{ref} in Fig. 14.6 – highlights the interest of using a LRM which is traceable to a CRM If this reference value is traceable the chart also provides information on drifts in trueness. The homogeneity and stability of LRMs is essential and can be verified in a similar manner as for CRMs (see Section 6).
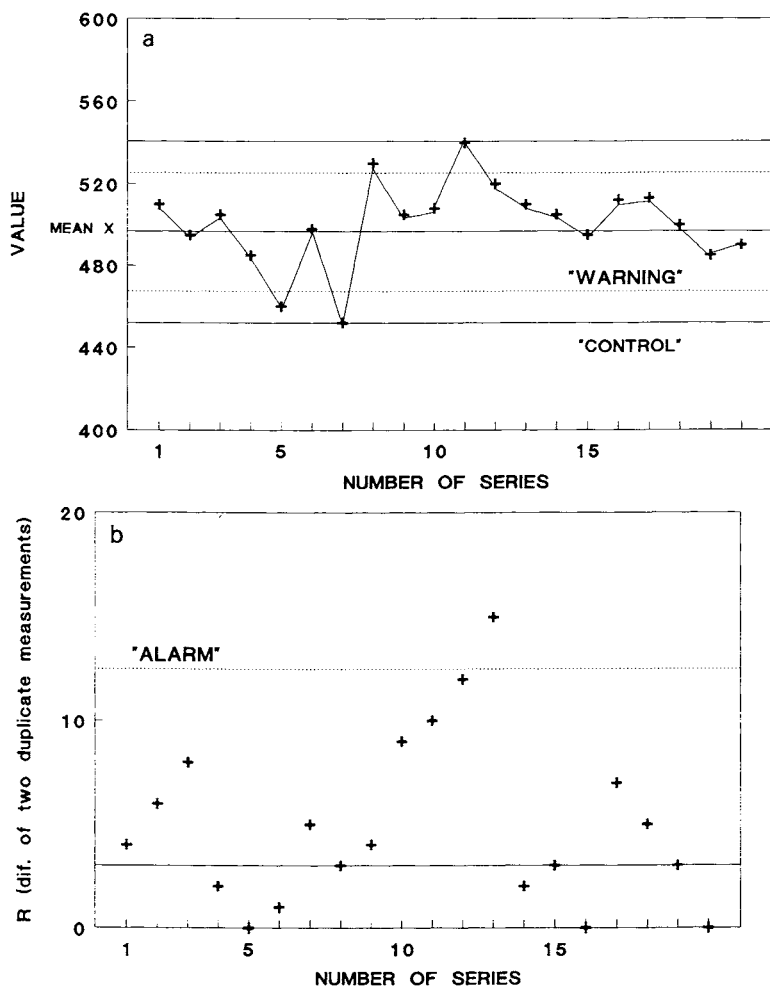
Fig. 14.5. Examples of X and R charts (arbitrary units). X is the value obtained at each occasion of analysis. R is the difference between two duplicate determinations. Warning and alarm lines correspond to a risk of 5% and 1%, respectively, that the result does not belong to the whole population of results.

The organisations producing CRMs would not normally become manufacturers of large quantities of LRMs. This is a task for industry or specialised servicing laboratories, as in the case of clinical chemistry [21], or should be done by each laboratory itself, possibly in collaboration with others.

14.5.4 Other uses of CRMs

CRMs can also serve the very useful purpose of demonstrating the equivalence of methods. This enables laboratories to follow the development of new analytical instrumentation. The analyst can compare the performance of his method with those from other

NUMBER OF SERIES
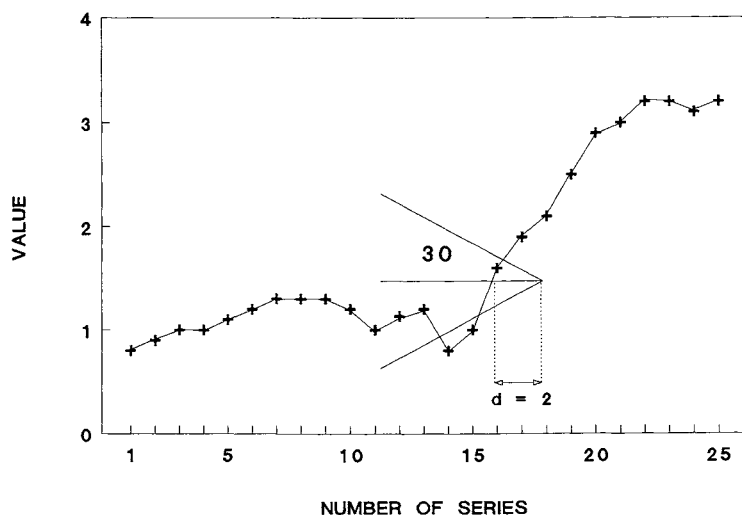
Fig. 14.6. Cusum chart. The sum of the differences between the found result and a reference value are plotted in time. They allow the detection of drifts and trends.

$$Y_i = \sum_{i=1}^{n} (X_i - X_{\text{ref}})$$

laboratories without the necessity for intercomparisons. Such use is essential when improvement of measurements is necessary and when comparability of rapidly evolving measurement technology is necessary in particular at an international level (e.g. preparation of European Community Directives).

Some methods, e.g. optical emission spectrometry, wavelength dispersive X-ray fluorescence, are calibrated with reference materials of a similar, fully characterised matrix (e.g. metal alloys). For such methods, accuracy can only be achieved when certified reference materials are used for the calibration.

This will not be developed here as it is not specific to environmental monitoring.

14.6 REQUIREMENTS FOR CERTIFIED REFERENCE MATERIALS

CRMs can be:

(a) pure substances or solutions to be used for calibration and/or identification;

(b) matrix reference materials which, as far as possible, represent the matrix being analysed by the user and which have a certified content (such materials are mainly to be used for the verification of a measurement process);

(c) materials of a known matrix composition for the calibration of a certain type of measuring instrument, e.g. spark source emission spectrometry, X-ray fluorescence (XRF), and those techniques which require a calibration with a material similar to the matrix analysed;

(d) methodologically-defined reference materials for parameters such as: leachable or aqua-regia-soluble fractions of trace elements from soils, ashes and slags; bio-availability

of a certain element, chloroform-extractable pesticides, etc.) the certified value is defined by the applied method following a very strict analytical protocol.

Attention will mainly be focused on matrix materials (category b).

CRMs have to fulfil many requirements. In order to assume properly their role in helping laboratories to achieve accuracy they should be as similar as possible to the unknown sample to be analysed. It is not possible to fulfil all requirements. Sometimes compromises are necessary; highly unstable compounds or matrices cannot be certified.

14.6.1 Selection of candidate CRMs

To verify the results of the analysis of a certain matrix, one should select a CRM which is similar to the unknown sample or which is at least as difficult. In most cases this means similarity of:

- the matrix composition;
- the contents of the analytes;
- how these analytes are bound;
- the fingerprint pattern of possible interference;
- the physical status of the material.

In preparing a candidate CRM these items should be taken into full consideration. In particular, for the speciation of, e.g. trace elements, or for the determination of organic compounds bound to the matrix, artificially spiked materials are not representative of real samples [27–29].

However, for practical reasons this similarity cannot always be respected. The material has to be homogeneous and stable in order to ensure that the samples delivered to the laboratories are identical. Therefore, compromises have to be made, and the preparation of the material has to be adapted.

14.6.2 Preparation of the material

The material has to be collected in a quantity sufficient to assure an adequate stock. CRMs should be available over several years to fulfil properly their task in the quality control and quality assurance system set up by the laboratories. Therefore, the quantity to be collected may be as much as 100 kg of solid material (e.g. soils or sediments) or several cubic meters (e.g. water CRMs). The producer needs to be properly equipped to treat such large amounts of material. The collected material has to be stabilised. This is one of the most sensitive and difficult steps in the work. The stabilisation has to be adapted to each particular case and should be studied in detail before processing in order to respect the integrity of the material as much as possible. Usually the materials are dried to avoid chemical or microbial changes. This may be achieved by heat or by freeze-drying, depending on the volatility of the analytes and matrix components. Some materials can be sterilised by gamma-irradiation (^{60}Co source). In reality this treatment is only possible for materials to be certified for inorganic parameters as many organic compounds are destroyed by gamma-irradiation [30]. Simple freezing of the material is also possible but the resulting material can only be used once as re-freezing may not lead to an homogeneous material. There is a tendency to produce more and more fresh materials. This concerns particular materials for environmental monitoring, e.g. target animal tissues,

plants, etc. In this case preservative substances have to be added to the matrix in order to avoid microbial growth. In 1999 the first fresh BCR material, PCBs in mussel tissue, will be available followed later on by fish tissue.

The material must also be homogenised and stored in adequate vials. For gases and liquids the achievement of homogeneity is not the most difficult problem; their stability, however, causes great concern. Solid materials are difficult to homogenise. When the particle size is lower than 125 mm and when the particle size distribution of the material is sufficiently narrow it has been shown that the homogeneity is sufficient even for sample intakes of less than 50 mg [31–35]. This can be achieved by a proper grinding procedure [36] and a thorough homogenisation before and during the filling procedure [37]. Unfortunately, the low particle size has its drawbacks as it leads to materials which are usually easier to analyse than real samples owing to better extractability of analytes [38] or easier acid or solvent attack on the matrix because of the large contact surface. Static electricity may cause sub-sampling difficulties for some materials with very low particle sizes and low water content [39].

14.6.3 Homogeneity and stability

A test portion of CRM in chemistry can be used only once when taken out of its storage vial. Therefore, the quantity of material in the bottle or ampoule should be sufficient to allow several determinations (20–50 g). A verification of the homogeneity has to be performed to ensure that, within a bottle or ampoule and from one vial to another, the content is the same (within- and between-vial homogeneity). The producer of the CRM should verify the minimum sample size for which the homogeneity is sufficient, i.e. for which he can guarantee that the uncertainty caused by inhomogeneity does not exceed the uncertainty of the certified values [40–42]. Usually, matrix materials segregate during transport. Therefore, in addition to the homogeneity data, the producer of the CRM should supply the user with sufficient information on how to re-homogenise the sample.

The producer of CRMs also should guarantee that the characteristics of the material and the certified parameters remain unchanged over longer periods. He should study beforehand the long-term stability to assess the bench life and possible damage during transport. The stability can be estimated by evaluating the behaviour of the material under accelerated ageing conditions [30,43]. For potentially unstable materials, stability checks may continue at regular intervals after certification during the entire availability of the CRM.

14.6.4 Certification

The certified value should be an accurate estimate of the true value with a reliable estimate of the uncertainty. The ISO Guide 35–1985 [44] gives several technically valid approaches for certifying a reference material. The Guidelines for the production and certification of BCR reference materials issued by the services of the European Commission give a more practical and complete approach as it is adapted to all types of chemical measurements and materials [45]. Depending on the type of RM and parameter to be certified, there may be some differences in the approach applied. Only certification of matrix materials will be discussed here.

One method of certifying a parameter in a RM consists in using a so-called definitive method, e.g. isotope dilution mass spectrometry (IDMS) in inorganic trace analysis, in one

single laboratory [44–46]. However, this laboratory may have a bias and therefore the certified value may be wrong. This method of using the results from one method in a single laboratory does not give a fair estimate of the uncertainty as achievable by other, more classical methods. In addition, such 'definitive methods' hardly exist in organic trace analysis.

The other approach, more often adopted, consists in using several independent methods. Certification becomes possible and reliable when sufficient evidence is available that two or more independent methods give the same result. Each method should be applied in three or more laboratories of proven high quality. It is clear that the laboratories have to demonstrate beforehand and during the certification exercise, that they work with methods which have been validated in detail, and particularly that proper calibration is performed to avoid systematic errors. This includes the calibration of balances, volumetric glassware and other tools of relevance, the use of calibrants of adequate purity and of known stoichiometry [20] and of proper solvents and reagents. Special precautions need to be taken when all laboratories use the same calibrant, e.g. because only one supplier exists. Chemical reaction yields should be known accurately and all precautions should be taken to avoid losses (e.g. from the formation of insoluble or volatile compounds, or incomplete extraction) and contamination.

If the results from entirely different methods, such as IDMS, INAA, ICP-MS, ICP-OES or atomic absorption spectrometry (AAS), and differential pulse anodic stripping voltammetry (DPASV) (between-method bias), as applied in different laboratories working independently (between-laboratory bias), are in agreement it can be concluded that the bias of each method is negligible and the mean value of the results is the best approximation to the true value. The remaining differences between laboratories are considered to be representative of the different sources of inaccuracy which still exist in the state of the art, and form a reliable basis for the determination of the uncertainty of the certified value.

For materials certified for living organisms, e.g. microbes, the interlaboratory approach has been successfully used by BCR. Several microbes have been certified in a survival medium (spray-dried milk powder) and can be spiked into the matrix to be monitored, e.g. water or food. These certifications have been performed following prescribed methods (ISO methods mainly) and measurements were performed using a detailed analytical protocol. The statistical treatment of data has been adapted to these special cases were a small limited number of particles are certified in the matrix [47]. Examples are given in Table 14.1.

14.6.5 Availability

Certified reference materials can really fulfil their task when they can be introduced into the quality assurance and quality control schemes set up by the laboratories. This can only be achieved when the analysts know that the CRMs will be available over long periods of time. The long term availability should oblige the producers to foresee batches of CRMs which are large enough to be available over several years and to replace exhausted CRMs by similar materials. However, replacement of CRMs is not easy as the freshly collected material may differ in composition from the previous CRM. An example of this situation has been encountered recently by the BCR (Bureau Communautaire de Référence) when it was necessary to replace a sewage sludge material certified for toxic elements (CRM 145).

TABLE 14.1

EXAMPLES OF SOME RECENT CRMS FOR THE QUALITY CONTROL OF MEASUREMENTS
IN ENVIRONMENTAL MONITORING (NON-NUCLEAR FIELD AND ONLY CERTIFIED MATRIX
MATERIALS)[a]

Type	CRM No.	Certified parameter	Supplier	Reference
Sediments				
Estuary	CRM 277R	Trace elements	BCR	[51]
Lake	CRM 280R	Trace elements	BCR	[51]
River	CRM 320	Trace elements	BCR	[51]
Lake	CRM 601	Extractable elements	BCR	[51]
Harbour	CRM 424	TBT	BCR	[51]
Estuary	CRM 580	Hg and MeHg	BCR	[51]
River-harbour	CRM 535	PAH	BCR	[51]
River-harbour	CRM 536	PCB	BCR	[51]
Antarctic	MURST-ISS-1	Trace elements	BCR/ISS	[51]
Estuary	SRM 1646	Trace elements	NIST	[46]
River	SRM 2704a	Trace and major elements	NIST	[46]
River	SRM 1939a	PCB	NIST	[46]
Marine	SRM 1941a	PAH-OCP-PCB	NIST	[46]
Waterway	SRM 1944	BeP	NIST	[46]
Marine	HS 3-6	PAH	NRCC	[49]
Marine	CS-1	Aroclor	NRCC	[49]
Harbour	HS 1-2	PCB	NRCC	[49]
Harbour	PACS-1	TBT + trace elements	NRCC	[49]
Marine	BCSS-1	Trace and major elements	NRCC	[49]
Estuary	MESS-1	Trace and major elements	NRCC	[49]
Estuary	BEST-1	Hg	NRCC	[49]
Harbour	EC-1	PAH	NWRI	[49]
Lake	EC-2	CB-PAH	NWRI	[49]
River	EC-3	CB-PAH	NWRI	[49]
River	HR 1	Trace and major elements	NWRI	[49]
Lake	WQB 1-2-3	Trace elements	NWRI	[49]
Stream	GBW07-309 to 07-312	Trace and major elements	NRC	[49]
Stream	GDS 10–12	Trace and major elements	NRC	[49]
Marine	GBW 07313	Trace and major elements	NRC	[49]
River	GSD 9	Trace and major elements	NRC	[49]
River	GBW 08301	Trace elements	NRC	[49]
Pond	NIES 2	Trace and major elements	NIES	[49]
Stream	SARM 46,51,52	Trace and major elements	SABS	[49]
Soils				
Loam	CRM 141R	Trace elements	BCR	[51]
Sandy	CRM 142R	Trace elements	BCR	[51]
Amended	CRM 143R	Trace elements	BCR	[51]
Amended	CRM 483–484	Extractable elements	BCR	[51]
Calcareous	CRM 600	Extractable elements	BCR	[51]
Industrial	CRM 524	PAH-PCP	BCR	[51]
Industrial	CRM 481	PCB	BCR	[51]

TABLE 14.1 (*continued*)

Type	CRM No.	Certified parameter	Supplier	Reference
Industrial	CRM 529–530	PCDD/F-CB-CP	BCR	[51]
San Joaquin	SRM 2709	Trace elements	NIST	[46]
Montana	SRM 2710-2711	Trace elements	NIST	[46]
No inf.	EPA SRS003-50	Trace elements	US-EPA	[49]
Industrial	EPA SRS103-100	PAH	US-EPA	[49]
No inf.	GBW 07401 to 07408	Trace and major elements	NRC	[49]
Tibet (no inf.)	GBW 08302	Trace and major elements	NRC	[49]
Farmland	GBW 08303	Trace and major elements	NRC	[49]
Brown	GSS-1	Trace and major elements	NRC	[49]
Desert	GSS-2	Trace and major elements	NRC	[49]
Yellow-brown	GSS-3	Trace and major elements	NRC	[49]
Yellow	GSS-4	Trace and major elements	NRC	[49]
Yellow-red	GSS-5–6	Trace and major elements	NRC	[49]
Laterite	GSS-7	Trace and major elements	NRC	[49]
Loess	GSS-8	Trace and major elements	NRC	[49]
Sediment rich	SARM 42	Major elements	SABS	[49]
Sewage sludge				
Domestic	CRM 144R	Trace elements	BCR	[51]
Mixed origin	CRM 145R	Trace elements	BCR	[51]
Industrial	CRM 146R	Trace elements	BCR	[51]
Mixed origin	CRM 392	PCB	BCR	[51]
Mixed origin	CRM 088	PAH	BCR	[51]
Mixed origin	CRM 597	Cr	BCR	[51]
Mixed origin	CRM 677	PCDD–PCDF	BCR	[51]
Separator sludge	EPA-SRS101–100	PAH	US-EPA	[49]
Ashes and dust				
Coal fly ash	CRM 038	Trace elements	BCR	[51]
Incineration fly ash	CRM 176	Trace elements	BCR	[51]
Incineration fly ash	CRM 490	PCDD–PCDF	BCR	[51]
Fly ash on filters	CRM 128	Trace elements	BCR	[51]
Welding dust	CRM 545	CrVI and leachable Cr	BCR	[51]
Coal fly ash	SRM 1633b	Trace elements	NIST	[46]
Coal fly ash	SRM 2689-91	Major and trace elements	NIST	[46]
Urban dust	SRM 1648	Trace elements	NIST	[46]
Urban dust	SRM 1649a	PAH	NIST	[46]
Diesel particulate	SRM 1650a	PAH	NIST	[46]

TABLE 14.1 (*continued*)

Type	CRM No.	Certified parameter	Supplier	Reference
Diesel particulate extract	SRM 1975	PAH	NIST	[46]
Diesel particulate	SRM 2975	PAH	NIST	[46]
Fly ash	EPA-SRS 001-100	Trace elements	US-EPA	[49]
Incineration ash	EPA-SRS 019-50	Trace elements	US-EPA	[49]
Incineration ash	EPA-SRS 203-225	Trace elements	US-EPA	[49]
Coal fly ash	GBW 08401-08402	Trace elements	NRC	[49]
Vehicle exhaust particulate	NIES 8	Trace elements	NIES	[49]
Waters				
Fresh water	CRM 398	Trace and major compounds	BCR	[51]
Fresh water	CRM 399	Trace and major compounds	BCR	[51]
Sea water	CRM 403	Trace elements	BCR	[51]
Sea water	CRM 179	Hg	BCR	[51]
Estuary	CRM 505	Hg	BCR	[51]
Ground water	CRM 609-610	Hg	BCR	[51]
Ground water	CRM 611-612	Bromide	BCR	[51]
Ground water	CRM 616-617	Major elements	BCR	[51]
Simulated rain water	CRM 408-9	Major compounds	BCR	[51]
Fresh water	CRM 479-480	nitrates	BCR	[51]
Fresh water lyoph.	CRM 606	Polar pesticides	BCR	[51]
Milk powder*	CRM 506	*Enterococcus faecium*	BCR	[51]
Milk powder	CRM 527	*Enterobacter cloacae*	BCR	[51]
Milk powder	CRM 594	*Escherichia coli*	BCR	[51]
Water	SRM 1643d	Trace elements	NIST	[46]
Natural water	SRM 1640	Trace elements	NIST	[46]
Water	SRM 1641	Hg	NIST	[46]
Simulated rain water	SRM 2694b	Major parameters	NIST	[46]
Near-shore sea water	CASS-2	Trace elements	NRCC	[49]
Open ocean water	NASS-4	Trace elements	NRCC	[49]
Estuary water	SLEW-1	Trace elements	NRCC	[49]
River water	SLRS-2	Trace elements	NRCC	[49]
River water	ORMS-1	Hg	NRCC	[49]

TABLE 14.1 (continued)

Type	CRM No.	Certified parameter	Supplier	Reference
Water	V-SMOW and SLAP	O&H stable isotope ratios	IAEA	[49]
Sea water	IAPSO	Conductivity	OSI	[49]
Waste				
Mineral oils	CRM 420-449	PCB	BCR	[51]
Shale oil	SRM 1580	PAH-phenols	NIST	[46]
Motor and transformer oil	SRM 1581	Aroclor	NIST	[46]
Crude oil	SRM 1582	PAH-phenols	NIST	[46]
Gases				
On Tenax	CRM 112–562	Benzene, toluene, *m*-xylene	BCR	[51]
Permeation tubes	SRM 1625-26	SO_2	NIST	[46]
Permeation tubes	SRM 1629a	NO_2	NIST	[46]
Air	SRM 1658a–69b and 2764/2750–51	CH_4–C_3H_8	NIST	[46]
Air	SRM 1671–72	CO_2	NIST	[46]
Air	SRM 2607–10	CO_2–NO_2	NIST	[46]
Air	SRM 2656–2660	NO_x	NIST	[46]
Air	SRM 2612–14	CO	NIST	[46]
Permeation tubes	GBW 08201	SO_2	NRC	[49]
Permeation tubes	GBW 08202	NO_2	NRC	[49]
Permeation tubes	GBW 08203	H_2S	NRC	[49]
Permeation tubes	GBW 08204	NH_3	NRC	[49]
Permeation tubes	GBW 08205	Cl_2	NRC	[49]
Air	GBW 08119	CH_4	NRC	[49]
Air	GBW 08120	CO_2	NRC	[49]
Air	GBW 08123	CH_4	NRC	[49]
Animal tissues				
Mussel tissue	CRM 278	Trace elements	BCR	[51]
Cod muscle	CRM 422	Trace elements	BCR	[51]
Milk powder	CRM 063R, 150–151	Trace elements	BCR	[51]
Human hair	CRM 397	Trace elements	BCR	[51]
Plankton	CRM 414	Trace elements	BCR	[51]
Tuna fish tissue	CRM 627	Forms of As	BCR	[51]

TABLE 14.1 (*continued*)

Type	CRM No.	Certified parameter	Supplier	Reference
Tuna fish tissue	CRM 463-464	MeHg + Hg	BCR	[51]
Mussel tissue	CRM 477	TBT, DBT, MBT	BCR	[51]
Cod liver oil	CRM 349	PCB	BCR	[51]
Mackerel oil	CRM 350	PCB	BCR	[51]
Milk powder	CRM 450	PCB	BCR	[51]
Milk powder	CRM 607	PCDD–PCDF	BCR	[51]
Pork fat	CRM 430	OCP	BCR	[51]
Cod liver oil	CRM 598	OCP	BCR	[51]
Milk powder	CRM 187–188	OCP	BCR	[51]
Animal feed	CRM 115	OCP	BCR	[51]
Coconut oil	CRM 458–459	PAH	BCR	[51]
Oyster tissue	SRM 1566b	Trace elements	NIST	[46]
Milk powder	SRM 1549-8435	Trace elements	NIST	[46]
Bovine serum	SRM 1598	Trace elements	NIST	[46]
Cow blood	SRM 955b	Pb	NIST	[46]
Cod liver oil	SRM 1588	PCB + OCP	NIST	[46]
Mussel tissue	SRM 1974a	PAH	NIST	[46]
Human serum	SRM 1589a	Aroclor	NIST	[46]
Whale blubber	SRM 1945	OCP–PCB	NIST	[46]
Mussel tissue	SRM 2974	OCP–PCB	NIST	[46]
Lobster hepato.	TORT-1	Trace elements	NRCC	[49]
Lobster hepato.	LUTS-1	Trace elements	NRCC	[49]
Dogfish liver	DOLT-1	Trace elements	NRCC	[49]
Dogfish muscle	DORM-1	Trace elements	NRCC	[49]
Mussel tissue	MUS-1	Domoic acid	NRCC	[49]
Fish tissue	EPA-SRS903	chlordane–OCP	US-EPA	[49]
Mussel tissue	GBW 08571	Trace elements	NRC	[49]
Prawn	GBW 08572	Trace elements	NRC	[49]
Sea bass tissue	NIES 11	Sn, TBT	NIES	[49]
Plant tissues				
Aquatic plants	CRM 060-061	Trace elements	BCR	[51]
Aquatic plant	CRM 596	Cr	BCR	[51]
Marine	CRM 279	Trace elements	BCR	[51]
Olive leaves	CRM 062	Trace elements	BCR	[51]
Hay powder	CRM 129	Trace elements	BCR	[51]
Rye grass	CRM 281	Trace elements	BCR	[51]
White clover	CRM 402	Trace elements	BCR	[51]
Lichens	CRM 482	Trace elements	BCR	[51]

TABLE 14.1 (*continued*)

Type	CRM No.	Certified parameter	Supplier	Reference
Spruce needles	CRM 101	Nutrients and contaminants	BCR	[51]
Beech leaves	CRM 100	Nutrients and contaminants	BCR	[51]
Citrus leaves	SRM 1572	Trace elements	NIST	[46]
Pine needles	SRM 1575	Trace elements	NIST	[46]
Apple leaves	SRM 1515	Trace elements	NIST	[46]
Peach leaves	SRM 1547	Trace elements	NIST	[46]
Spinach leaves	SRM 1570a	Trace elements	NIST	[46]
Tomato leaves	SRM 1573a	Trace elements	NIST	[46]
Vegetation	SRM 2695	Fluoride	NIST	[46]
Sargasso seaweed	NIES-9	Trace elements	NIES	[49]

[a] Abbreviations used in the table are as follows. *Substances*: BeP, Benzo(*e*)pyrene; CB, chlorobenzenes; CP, chlorophenols; MeHg, methyl mercury; OCP, organochlorine pesticides; PAH, polycyclic aromatic hydrocarbons; PCDD–PCDF, polychlorodibenzo-*p*-dioxins and polychlorodibenzo-furans; PCP, pentachlorophenol; TBT, DBT, MBT, tri-, di-, mono-butyl tin. *Miscellaneous:* part., particulate matter; milk powder*, capsules to be dissolved into water; hepato., hepatopancreas; Lyoph., lyophilised. *CRM producers*: ISS, Istituto Superiore de la Sanita, Rome, Italy; NIES, National Institute for Environmental Studies of Japan, Tsukuba, Japan; NRC, National Research Centre for CRM, Beijing, China; NWRI, National Water Research Institute, Burlington, Canada; OSI, Ocean Scientific International Ltd., Wormley, Surrey, UK; SABS, South Africa Bureau of Standards, Pretoria, RSA; US-EPA, United States Environmental Protection Agency, Cincinnati, USA.

It was not possible to find a material with exactly the same trace element pattern. In particular, the Ni and the Cd contents in the prospective materials were always much lower than in the previous CRM 145, which was collected 15 years before. This may reflect the actual contamination of sewage sludge by these elements. Therefore the new material may better reflect the situation encountered with natural samples analysed by the laboratories.

14.7 CRMs for environmental monitoring

14.7.1 Suppliers

There are a number of suppliers of CRMs for environmental monitoring. Two main bodies, the National Institute of Standards and Technology (NIST, USA) and the BCR of the European Commission, cover several fields and ensure long-term availability of CRMs, owing to the large batches of materials produced. The International Atomic Energy Agency (IAEA) in Vienna (Austria) mainly provides certified materials for nuclear measurements but also has available some CRMs for non nuclear analysis. The ISO Council on Reference Materials (REMCO) of ISO has available a Directory for Reference Materials which may be consulted [48]. Some additional compilations of existing CRMs in more specialised fields also exist [49]. The major source of information on reference

materials is the COMAR Data Bank which can be consulted through several national metrology institutes.

14.7.2 Types of CRMs

CRMs are products of very high added value. Their production is very costly (some hundred thousands of Euro) and therefore they should be reserved for selected tests as final verification of the analytical procedure. The CRMs for environmental monitoring mainly concern matrix materials certified for chemical or biochemical content, but sometimes physical parameters (e.g. the conductance of rainwater – NIST SRM 2694). Reference materials certified for microbiological parameters are being prepared by the BCR [50], mainly for the quality control of microbiological determinations in water and food.

Table 14.1 gives a list of some CRMs already available from various suppliers in 1997. This list covers only matrix materials in the non-nuclear field and is not exhaustive. For more updated information on the CRMs the user should contact the producers [46,49,51]. Some animal tissues or products or plants are listed here as they are often used to evaluate the global contamination of the environment (target animals or plants) or because they are representative of the contamination in the food chain.

Several industrial products, e.g. coals, may serve for the economic evaluation of materials as well as for the control of potential contamination by some toxic compounds or elements. Therefore, they have some interest for environmental monitoring but have not been listed in Table 14.1.

14.8 CONCLUSION

Certified reference materials are keys to the achievement of reliable, accurate results which are the basis of the proper monitoring of the environment and consequently for proper decisions to be taken to maintain or improve the prevailing situation. The use of CRMs in laboratories ensures the possibility of providing accurate results, which are also traceable to recognised international references (standards) and therefore can be compared with the results from any other laboratory having the same traceability.

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Chapter 15

Standard reference materials for the determination of trace organic constituents in environmental samples

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CONTENTS

15.1 INTRODUCTION

The quality of analytical measurements for the determination of organic contaminants in the environment depends to a large extent on the availability and use of certified

reference materials (CRMs) to validate the analytical procedures and to demonstrate that the measurement process is in control. Ideally, the CRMs should be natural environmental matrices that are similar to the sample matrix to be analyzed with appropriate levels of the compounds of interest. For nearly two decades the National Institute of Standards and Technology (NIST) has been involved in the development of Standard Reference Materials® (SRMs®), which are NIST-issued CRMs, for the determination of organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and chlorinated pesticides in natural environmental matrices such as fossil fuels [1,2], air and diesel particulate material [3–5], coal tar [6], sediment [7–9], mussel tissue [10–12], fish oil [13], and whale blubber [14]. Several papers have reviewed and summarized the development of these environmental matrix SRMs [15–18] including the first volume of this book in 1993 [17]. This chapter is intended as an update (since the 1993 publication) of the activities at NIST related to the continuing development of SRMs for organic contaminants in environmental samples.

In the 1993 review [17] we described in detail the first decade of activities at NIST related to the development of environmental SRMs for organic contaminant measurements including a detailed description of the various SRMs and the development of the basic analytical approach used to assign certified values. In this chapter we describe the 'second' generation of environmental SRMs, particularly the natural matrix materials, and the improvements in the analytical approach used for value assignment. The various modes of value assignment of SRMs for chemical composition at NIST have recently been reexamined and are described briefly in this chapter with examples to illustrate their use. Finally, the recent activities in SRM development for the past six years are described including recertifications of existing materials, renewals of previous materials, and the development of new materials.

15.2 MODES OF CERTIFICATION

Historically, NIST has used three basic modes for 'certification' of chemical composition for SRMs: (1) measurements using a 'definitive or primary' method, i.e., a method of high precision for which all sources of bias have been rigorously investigated [19]; (2) measurements using two or more independent and reliable methods; and (3) measurements from a number of laboratories participating in a multi-laboratory comparison exercise, i.e., a round robin study. The basic approach of these modes as specifically applied to the value assignment for inorganic constituents in natural matrices has been described previously [19,20]. Recently NIST reexamined the various approaches used for value assignment of chemical composition in SRMs and clearly defined the terms used to describe the composition values assigned to the SRMs [21]. Three types of assigned values were defined: certified, reference, and information [21]. These three terms are defined in Table 15.1. The above-mentioned three historical approaches to certification were expanded to identify seven modes that are used at NIST for value assignment for chemical composition. These seven modes and the resulting values are summarized in Table 15.2. The basic principles of value assignment remain unchanged; however, these modes now provide a well-defined link between the process used for value assignment and the definition of the assigned value (i.e., certified, reference, or information value). The terms in Table 15.1 provide a clear

TABLE 15.1

DEFINITION OF TERMS USED FOR NIST SRMs AND RMs FOR CHEMICAL COMPOSITION

Certified value
A value reported on an SRM Certificate of Analysis for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been fully investigated or accounted for by NIST. The value has an associated uncertainty that generally specifies a range within which the true value is expected to lie at a level of confidence of approximately 95% if the sample is homogeneous or the uncertainty represents a prediction interval within which the true values of 95% of all samples are expected to lie at a stated level of confidence if significant sample heterogeneity is included

Reference value
A value reported on an SRM Certificate of Analysis and/or a Reference Material (RM) Report of Investigation that represents the best estimate of the true value where all known or suspected sources of bias have not been fully investigated by NIST. The value has an associated uncertainty that may not include all sources of uncertainty and may represent only a measure of the precision of the measurement method(s)

Information value
A value that will be of interest and use to the SRM/RM user, but insufficient information is available to NIST to assess adequately the uncertainty associated with the value. Typically the value will be provided with no uncertainty listed

indication of the level of confidence that NIST has in the accuracy of the assigned value. The definition of a certified value in Table 15.1 implies that NIST must be involved in the measurement process for the value to be classified as a certified value (see modes 1–3). Thus, modes 4 and 7, which do not involve NIST measurements, result in reference values even though these modes may involve independent analytical methods. In some cases the reference values may be of the same quality relative to the accuracy as a certified value; however, because all sources of bias have not been thoroughly investigated by NIST, the value is designated as a reference value. Several other organizations that provide CRMs use results from interlaboratory exercises to assign certified values to their natural matrix materials (mode 7 in Table 15.2). In the NIST definition of terms such values are denoted as reference values to indicate that NIST was not involved in making any measurements that contributed to the value assignment.

The typical mode used at NIST for certification of natural matrix SRMs for organic contaminants has been the analysis of the material using two or more 'chemically independent' analytical techniques (mode 2 in Table 15.2). The results of these multiple technique analyses, if in agreement, are used to determine the certified concentrations for the measured analytes. The requirement for using two or more analytical techniques is based on the assumption that the agreement of the results from the independent methods minimizes the possibility of biases within the analytical methods. When results are obtained from only one analytical technique (or multiple techniques that are not sufficiently independent), the concentrations are typically reported as reference values (mode 6 in Table 15.2) and are considered as a best estimate of the true value where all known or

TABLE 15.2

MODES USED AT NIST FOR VALUE ASSIGNMENT OF REFERENCE MATERIALS FOR CHEMICAL COMPOSITION

Mode of value assignment	Certified value	Reference value	Information value
1. Certification at NIST using a Primary Method with confirmation by other method(s)	X		
2. Certification/value assignment at NIST using two independent, critically-evaluated methods	X	X	
3. Certification/value assignment using one method at NIST and different methods by outside collaborating laboratories	X	X	
4. Value assignment based on measurement of two or more outside collaborating laboratories using different methods		X	X
5. Value assignment based on a method dependent (procedurally-defined) technique		X	X
6. Value assignment based on NIST measurements by a single method (but does not meet the criteria for certification) and/or outside collaborating laboratory measurements using a single method		X	X
7. Value assignment based on selected data from interlaboratory studies		X	X

suspected sources of bias have not been fully investigated by NIST. As the need increases for more natural matrix SRMs with values assigned for more analytes, NIST will rely more on data provided by outside laboratories (modes 3, 4, and 7 in Table 15.2) to maximize its resources. Recent certifications of natural matrix SRMs have included the use of selected results from multilaboratory comparison exercises as an additional data set in the assignment of the certified and reference values.

15.3 TYPES OF ENVIRONMENTAL SRMs

SRMs are used primarily for the following purposes: (1) to calibrate the measurement system, (2) to validate the accuracy and precision of a new analytical method, and (3) to provide quality control of routine analyses by analyzing the SRM at appropriate, regular time intervals. The current NIST SRMs for organic environmental analyses are summarized in Tables 15.3 and 15.4. The SRMs in Tables 15.3 and 15.4 represent two types of

TABLE 15.3

NIST CALIBRATION SOLUTION SRMs FOR THE DETERMINATION OF ORGANIC CONTAMINANTS

SRM No.	Title	Date issued	Certified values	Reference (non-certified) values
1491	Aromatic hydrocarbons in hexane/toluene	1989	PAHs (23)	PAHs (1)
1492	Chlorinated pesticides in hexane	1992	Pesticides (15)	
1493	Chlorinated biphenyl congeners in 2,2,4-trimethylpentane	1993	PCBs (18)	PCBs (2)
1494	Aliphatic hydrocarbons in 2,2,4-trimethylpentane	1993	Hydrocarbons (20)	
1584	Priority pollutant phenols in methanol	1984	Phenols (10)	Phenols (1)
1586	Isotopically labeled and unlabeled priority pollutants in methanol	1984	Priority pollutants (10)	
1587	Nitrated PAHs in methanol	1985	Nitro-PAHs (6)	Nitro-PAHs (1)
1596	Dinitropyrene isomers and 1-nitropyrene in methylene chloride	1987	Nitro-PAHs (4)	
1614	Dioxin (2,3,7,8-TCDD) in iso-octane	1985	Dioxins (2)	Dioxins (2)
1639	Halocarbons (in methanol) for water analysis	1983	Halocarbons (7)	
1647d	Priority pollutant PAHs (in acetonitrile)	1996	PAHs (16)	
2260	Aromatic hydrocarbons in toluene (nominal concentration 60 µg/ml)	1991	PAHs (23)	PAHs (1)
2261	Chlorinated pesticides in hexane (nominal concentration 2 µg/ml)	1992	Pesticides (15)	
2262	Chlorinated biphenyl congeners in 2,2,4-trimethylpentane (nominal concentration 2 µg/ml)	1993	PCBs (25)	PCBs (3)
2269	Perdeuterated PAH in toluene I	In prep.	Perdeuterated PAHs (5)	
2270	Perdeuterated PAH in toluene II	In prep.	Perdeuterated PAHs (6)	
2274	PCB congeners in 2,2,4-trimethylpentane (II)	In prep.	PCBs (11)	
2275	Chlorinated pesticides in 2,2,4-trimethylpentane (II)	In prep.	Pesticides (9)	
2276	Planar PCB congeners in 2,2,4-trimethylpentane	In prep.	PCBs (3)	

TABLE 15.4

RECENT NIST NATURAL MATRIX SRMs FOR THE DETERMINATION OF ORGANIC CONTAMINANTS IN ENVIRONMENTAL SAMPLES

SRM No.	Title	Date issued	Certified values	Reference (noncertified) values
1588a	Organics in cod liver oil	1998[a]	PCBs (24); pesticides (14)	PCBs (34); pesticides (3)
1589a	PCBs in human serum	In prep.	PCBs and pesticides	
1649a	Urban dust	1998[b]	PAHs (22); PCBs (35); pesticides (8)	PAHs (22); pesticide (1); PCDDs/PCDFs (17)
1650a	Diesel particulate matter	1999[c]	PAHs (19); Nitro-PAHs (1)	PAHs (25); Nitro-PAHs (3)
1939a	PCB congeners in river sediment	1998[d]	PCBs (20); pesticides (3)	PCBs (4)
1941a	Organics in marine sediment	1994[e]	PAHs (23) PCBs (21); pesticides (6)	PAHs (14); PCBs (7); pesticides (4); trace elements (27)
1944	NY/NJ waterway sediment	1999	PAHs (24); PCBs (35); pesticides (4); trace elements (9)	PAHs (32); PCDDs/PCDFs (17); pesticides (7); trace elements (20)
1945	Organics in whale blubber	1994	PCBs (27); pesticides (15)	PCBs (2); pesticides (2)
1974a	Organics in mussel tissue	1995[f]	PAHs (15); PCBs (20); pesticides (7); Methyl-Hg	PAHs (18); PCBs (4); pesticides (4); trace elements (32)
1975	Diesel particulate extract	1999	PAHs (8)	PAH (25) Nitro-PAHs (15)
2974	Organics in freeze-dried mussel tissue	1997	PAHs (14); PCBs (20); pesticides (7); Methyl-Hg	PAHs (17); PCBs (4); pesticides (4); trace elements (32)
2975	Diesel particulate matter (industrial forklift)	1999	PAHs (11)	PAHs (~25)
2977	Mussel tissue (organic contaminants and trace elements)	1999	PAHs (14); PCBs (25); pesticides (7); trace elements (6); Methyl-Hg	PAHs (16); trace elements (9)
2978	Mussel Tissue (organic contaminants - Raritan Bay, NJ)	1999	PAHs (7); PCBs (22); pesticides (12)	PAHs (20), PCBs (2)

[a] Originally issued in 1989; same material recertified in 1998.
[b] Originally issued in 1982; same material recertified in 1998.
[c] Originally issued in 1985; same material recertified in 1999.
[d] Originally issued in 1990; same material recertified in 1998.
[e] SRM 1941 first issued in 1989.
[f] SRM 1974 first issued in 1990.

materials with different levels of analytical difficulty and with different primary uses: (1) simple calibration solutions containing a number of analytes and (2) natural matrix materials with natural levels of contaminants (i.e., not fortified). The calibration solutions (see Table 15.3) are useful for validating the chromatographic separation step (e.g., retention times and analyte detector response); whereas the natural matrix materials (see Table 15.4), which are similar to the actual environmental samples analyzed, are used to validate the complete analytical procedure including extraction, isolation/cleanup procedures, and the final chromatographic separation, detection, and quantification. For each of the SRMs listed in Tables 15.3 and 15.4, information is provided on the number of analytes for which certified and reference concentration values are provided in the Certificate of Analysis that accompanies each SRM or the Report of Investigation that accompanies each Reference Material (RM).

15.3.1 Calibration solution SRMs

The calibration solution SRMs are useful for several purposes including: (1) calibration of chromatographic instrumentation for retention times and detector response factors for quantification, (2) spiking or fortifying samples, (3) analyte recovery studies, and (4) determining method (detector) response factors. The current NIST calibration solution SRMs are listed in Table 15.3. This list of materials has not changed significantly since the previous review, but is included here for completeness. Several calibration solution SRMs that were issued in the 1980s (e.g., SRM 1583 Chlorinated Pesticides in 2,2,4-Trimethyl-pentane and SRM 1585 Chlorinated Biphenyls in 2,2,4-Trimethylpentane) have since been replaced by other SRMs with more analytes (i.e., SRMs 1492 and 2261, Chlorinated Pesticides in Hexane, and SRMs 1493 and 2262, Chlorinated Biphenyl Congeners in 2,2,4-Trimethylpentane). The most recent activities related to calibration solution SRMs have focused on additional solutions containing additional PCB congeners and chlorinated pesticides (SRMs 2274, 2275, and 2276), aliphatic hydrocarbons (SRM 1494), and perdeuterated PAHs (SRMs 2269 and 2270); the latter for use as internal standards in the measurement of PAHs. SRM 1647, Priority Pollutant PAHs in Acetonitrile, one of the first (1981) and by far the most popular of the calibration solution SRMs, was recently reissued for the fourth time.

As part of a new program in support of the externalization of the U.S. Environmental Protection Agency's (EPA) Water Proficiency Testing studies, NIST is preparing a number of new calibration solution SRMs. These solution SRMs will include six different Aroclors in methanol and transformer oil; toxaphene and total chlordane in methanol; chlorinated herbicides in methanol; chlorinated pesticides in acetone; haloacetic acids in methyl-*t*-butyl ether; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in methanol; endothall, glypho-sphate, and diquat dibromide in water; chloral hydrate in methanol; carbamates in acet-onitrile; and adipate and phthalates in methanol.

15.3.2 Natural matrix SRMs

Validation of the complete analytical procedure (including solvent extraction, cleanup of the extracts, isolation of the analytes of interest, and chromatographic analysis) requires the use of SRMs with matrices similar to those typically encountered in the analysis of environmental samples. Thus, the natural matrix SRMs are the most suitable materials for

this purpose. In the previous review [17], we described the first natural matrix SRMs developed for the measurement of organic contaminants. To meet the increasing needs for more matrices and more analytes, most of the recent NIST SRM activities have focused on the updating of existing SRMs with additional information, providing replacement materials for previous SRMs (i.e., renewals), and developing new natural matrix SRMs. Seventeen natural matrix SRMs for the determination of organic contaminants are currently available from NIST with certified and reference concentrations primarily for PAHs, PCBs, chlorinated pesticides, polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). The significant developments since the previous review are described below. Table 15.4 lists 14 of the 17 natural matrix SRMs currently available; these 14 SRMs are the materials that have been issued or updated since the previous review.

15.4 ANALYTICAL APPROACH FOR THE CERTIFICATION OF NATURAL MATRIX SRMs

As stated above the approach to value assignment for organic constituents in environmental matrix SRMs at NIST is generally based on the use of two or more independent analytical techniques. The independence of the analytical techniques should be reflected in all aspects of the procedure including sample extraction, cleanup of the extract, and finally the separation, detection, and quantification of the analytes of interest. The first environmental matrix SRMs with certified values for PAHs were issued in the early 1980s using this basic approach with gas chromatography with flame ionization detection (GC–FID) or gas chromatography with mass spectrometric detection (GC/MS) and reversed-phase liquid chromatography with fluorescence detection (LC–FL) as the two principal analytical techniques [1–3,7,10]. The first natural matrix SRMs with certified values assigned for individual PCB congeners and pesticides were not issued until 1989 and 1990 (i.e., SRMs 1588 and 1939). The improvement of the analytical techniques and the evolution of the analytical approach for value assignment for PAHs, PCBs, and chlorinated pesticides from 1980 to 1993 was summarized in the previous volume of this book [17] with a brief update through 1997 published later [18]. In both of these previous publications, the basis of the independent techniques approach for PAHs, PCBs, and pesticides was discussed in detail. In this chapter the recent certification of SRM 1649a Urban Dust and SRM 1944 New York/New Jersey Waterway Sediment are described as examples of the current analytical approach used for the certification of PAHs, PCBs, and pesticides in an environmental matrix.

15.4.1 Analytical approach for determination of PAHs

For the determination of PAHs in the natural matrix SRMs listed in Table 15.4, various combinations of reversed-phase LC–FL and GC/MS have been used to provide the necessary two or more independent analytical techniques. For the natural matrix SRMs issued through the 1980s, NIST typically provided certified concentrations for only 5–12 PAHs; an additional 5–25 PAHs were often listed as noncertified (now denoted as reference) concentrations primarily because of the lack of measurements by the required second independent analytical technique [1–3,7,10]. These SRMs represent the 'first generation'

of natural matrix SRMs for organic contaminants developed at NIST. In the mid-1990s two renewal SRMs, SRM 1941a Organics in Marine Sediment [8] and SRM 1974a Organics in Mussel Tissue (*Mytilus edulis*) [11,12] were issued with a greater number of certified and reference values for PAHs. To provide value assignment for this greater number of PAHs, four different analytical techniques were implemented: (1) reversed-phase LC–FL analysis of the total PAH fraction, (2) reversed-phase LC–FL analysis of isomeric PAH fractions isolated by normal-phase LC (i.e., multidimensional LC), (3) GC/MS analysis of the PAH fraction on a 5% phenyl methylpolysiloxane stationary phase, which is the typical GC stationary phase used for PAH analyses, and (4) GC/MS of the PAH fraction on a smectic liquid crystalline stationary phase which provides excellent selectivity for the separation of PAH isomers. These techniques had been reported previously for the measurement of PAHs in several environmental SRMs [1–4,7,10]; however, not until the certification analyses of SRMs 1941a and 1974a were all four of these techniques used together to provide certified values for 23 and 15 PAHs, respectively. A detailed discussion of this approach has been reported for the certification of PAHs in SRM 1941a [8]. SRM 1941a and SRM 1974a were the first materials that could be considered as the second generation of natural matrix SRMs for organic contaminants at NIST. The basic analytical approach for the value assignment of these two SRMs was expanded recently for the recertification of SRMs 1649a and 1650a and the certification of the new marine sediment, SRM 1944, with the addition of a new extraction procedure, i.e., pressurized fluid extraction (PFE), and the use of a new GC stationary phase with different separation selectivity (50% phenyl methylpolysiloxane) compared to the 5% phenyl phase and the smectic liquid crystalline phase. The certification of SRM 1649a is described below in detail as an example of the current approach for determination of PAHs in environmental matrix SRMs.

The analytical scheme used for the certification measurements for PAHs in SRM 1649a is illustrated in Fig. 15.1. A total of seven sets of results from different methods for the determination of the PAHs were used for assignment of the certified values. These seven data sets are not completely independent of each other but are a combination of several independent procedures in each of the steps in the analytical process, i.e., extraction, cleanup, separation, and detection. The assumption is made that each independent procedure has different sources of bias (if biases exist); therefore, if the final results are in agreement, it suggests that none of the procedures have significant biases.

Two different solvent extraction procedures (Soxhlet extraction and PFE) with three different solvent systems (i.e., dichloromethane, 50% *n*-hexane/50% acetone, and acetonitrile) were used to extract the air particulate material (see Fig. 15.1). The traditional Soxhlet extraction procedure was used for five of the data sets and PFE, a recently developed alternative extraction procedure using high temperature (100–200 °C) and high pressure (1000–2200 psi; 6.90–15.2 MPa), was used for the remaining two sets of results. Prior to the incorporation of PFE as part of the certification measurements, Schantz et al. [22] evaluated and validated the use of PFE for the extraction of PAHs, PCBs, and pesticides from environmental matrices using several SRMs and CRMs including marine sediment, air and diesel particulate matter, mussel tissue, and fish tissue. In this study [22] they verified that the results obtained with PFE were comparable to those obtained using the traditional Soxhlet extraction procedure. Sample cleanup of the solvent extracts of SRM 1649a consisted of solid phase extraction (SPE) on an aminopropylsilane cartridge

SRM 1649a, Urban Dust

10 bottles (duplicates) 1 g	6 bottles 1 g	6 bottles 0.4 - 1 g	6 bottles 1 g	6 bottles 3 g
Soxhlet DCM	Soxhlet DCM	PFE DCM	PFE DCM, Hex/Ace, CH$_3$CN	Soxhlet 50% Hexane/ 50% Acetone
SPE	SPE	SPE	SPE	SPE
NPLC (aminopropylsilane) Total PAH	NPLC (aminopropylsilane)	NPLC (aminopropylsilane) Total PAH	NPLC (aminopropylsilane) Total PAH	NPLC (aminopropylsilane)
	Total PAH			Isomer Fractions
GC/MS (I) 5% phenyl methyl polysiloxane	GC/MS (sm) smectic liquid crystalline	GC/MS (II) 5% phenyl methyl polysiloxane	GC/MS (III) 50% phenyl methyl polysiloxane	GC/MS (IV) 5% phenyl methyl polysiloxane	LC-FL (Total)	LC-FL (Fractions)

Fig. 15.1. Analytical scheme for the certification of PAHs in SRM 1649a.

followed by either direct analysis (in the case of LC–FL (Total)) or further cleanup using normal-phase liquid chromatography (NPLC) on a semipreparative aminopropylsilane column to isolate either a total PAH fraction for GC/MS analysis or isomer fractions for LC–FL analysis (see Fig. 15.1).

The most significant differences (i.e., independence) in the analytical methods are provided in the final chromatographic separation and detection step using GC/MS and LC–FL. GC and reversed-phase LC provide significantly different separation mechanisms for PAHs and thus provide the independence required in the separation. The use of mass spectrometry (MS) for the GC detection and fluorescence spectroscopy for the LC detection provide further independence in the methods, e.g., MS can not differentiate among PAH isomers whereas fluorescence spectroscopy often can. For the GC/MS analyses the 5% phenyl methylpolysiloxane phase has been a commonly used phase for the separation of PAHs; however, several important PAH isomers are not completely resolved on this phase (see Fig. 15.2), i.e., chrysene and triphenylene, benzo[b]fluoranthene and benzo[j]-fluoranthene, and dibenz[a,h]anthracene and dibenz[a,c]anthracene. To achieve separation of these isomers, GC/MS analyses were also performed using two other phases with different selectivity, a 50% phenyl methylpolysiloxane phase [23] and a smectic liquid crystalline phase [24,25]. Both of these phases completely resolve the benzofluoranthene isomers (molecular weight 252) and dibenzanthracene isomers (molecular weight 278) (only partially resolved on the 50% phenyl phase); however, only the smectic liquid crystalline phase completely separates the isomeric triphenylene and chrysene (molecular weight 228). The separations of these three isomer groups on the different columns are illustrated in Fig. 15.2 for SRM 1649a.

The elution order of the PAHs on the 5% and 50% phenyl methylpolysiloxane phases is similar with only minor differences, e.g., benzo[j]fluoranthene elutes after benzo[k]fluoranthene among the 252 molecular weight isomers and pentaphene elutes before dibenz[a,c]anthracene and dibenz[a,h]anthracene among the 278 molecular weight isomers on
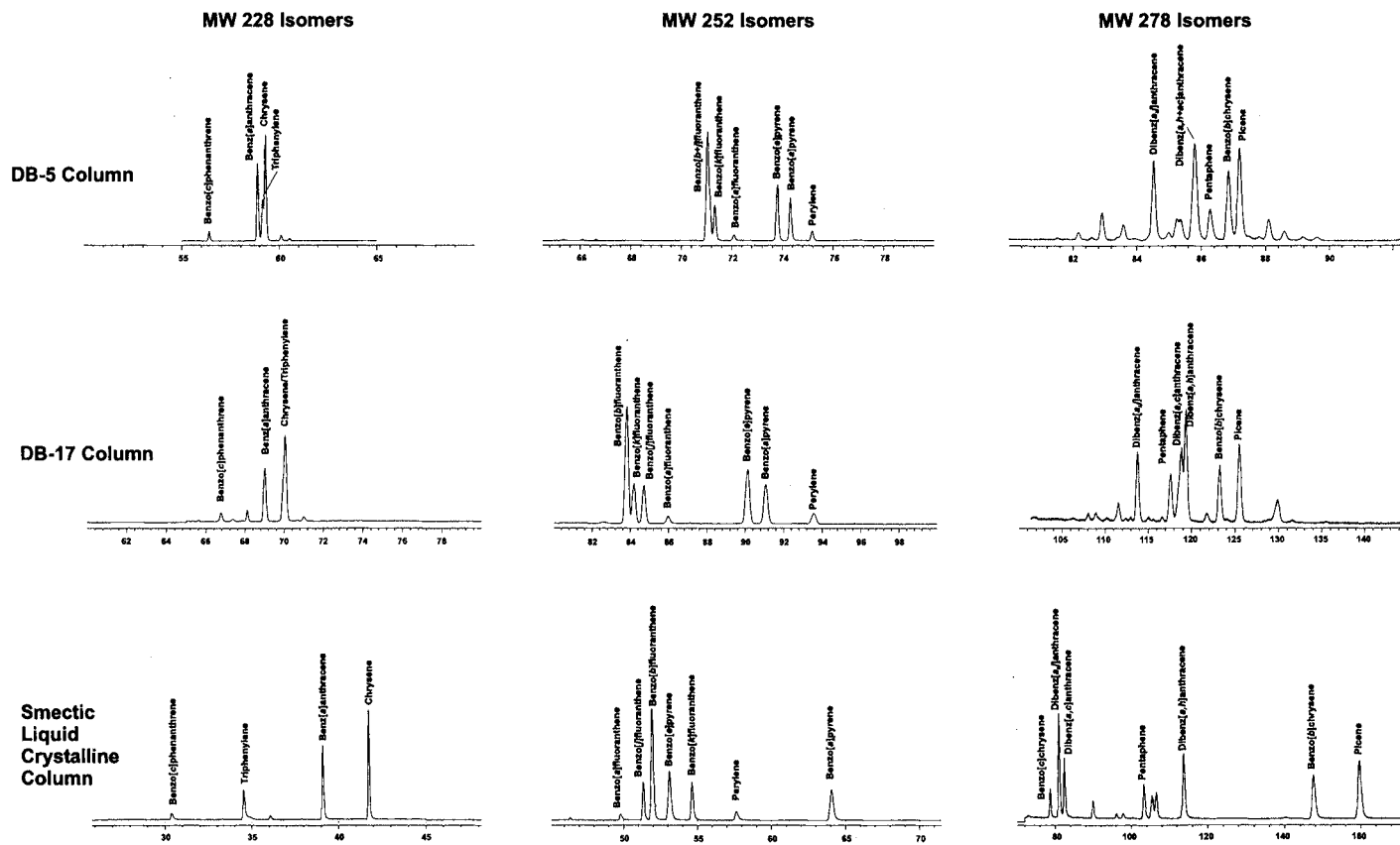
Fig. 15.2. GC/MS analysis of the total PAH fraction from SRM 1649a using three stationary phases for the determination of PAH isomers of molecular weights 228, 252, and 278: (A) 5% phenyl methylpolysiloxane, (B) 50% phenyl methylpolysiloxane and (C) smectic liquid crystalline phase.

the 50% phenyl phase. The smectic liquid crystalline phase provides a very different elution order compared with the two phenyl phases. The GC separation on the liquid crystalline phase is based to some extent on the shape of the PAHs, i.e., the more rod-like PAH isomers elute later than the more compact structures [24,25]. The difference in elution order for the liquid crystalline phase is most pronounced for the 252 molecular weight isomers where the elution order changes from benzo[*b*]fluoranthene, benzo[*k*]-fluoranthene, benzo[*j*]fluoranthene, benzo[*a*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, and perylene using the 50% phenyl phase to an elution order of benzo[*a*]fluoranthene, benzo[*j*]fluoranthene, benzo[*b*]fluoranthene, benzo[*e*]pyrene, benzo[*k*]fluoranthene, perylene, and benzo[*a*]pyrene using the liquid crystalline phase. For the 278 molecular weight isomers the elution order is similar but the selectivity (i.e., relative separation of the isomers) is much greater on the liquid crystalline phase. In general the selectivity, as well as the range over which an isomer group elutes, increase slightly from the 5% phenyl phase to the 50% phenyl phase, but increase significantly for the liquid crystalline phase. For example the elution ranges of the 252 molecular weight isomers on the 5% phenyl, 50% phenyl, and liquid crystalline phase are 4 min, 10 min, and 14 min, respectively. For the 278 molecular weight isomers the elution ranges for the three columns are 3 min, 13 min, and 84 min. The extremely long elution times for the 278 molecular weight isomers on the liquid crystalline phase make its use impractical for routine determination of PAHs. However, the unique selectivity of the liquid crystalline phase, and the differences in selectivity of the two phenyl phases provide the necessary independence among the GC/MS methods to justify the use of results from these three columns in the determination of the certified values for PAHs.

In the analysis of complex PAH mixtures obtained from environmental samples, reversed-phase LC–FL typically provides reliable results for only 8–12 major PAHs [26]. To increase the number of PAHs determined by LC–FL, a multidimensional LC procedure is used to isolate and enrich specific isomeric PAHs that could not be measured easily in the total PAH fraction because of interferences, low concentrations, and/or low fluorescence sensitivity or selectivity. This multidimensional procedure, which has been described previously [3,26,27], consists of a normal-phase LC separation of the PAHs based on the number of aromatic carbon atoms in the PAH, thereby providing fractions containing only isomeric PAHs and their alkyl-substituted isomers. These isomeric fractions are then analyzed by reversed-phase LC–FL to separate and quantify the various isomers. For the certification of SRM 1649a three isomer fractions were isolated and analyzed: the four aromatic ring *cata*-condensed PAHs (molecular weight 228), the five aromatic ring *cata*-condensed PAHs (molecular weight 278), and the six aromatic ring *peri*-condensed PAHs (molecular weight 276).

The reversed-phase LC analyses (both total and isomer fractions) are performed using a polymeric C_{18} phase, which provides excellent selectivity for the separation of PAH isomers [28–30]. Wavelength programmed fluorescence detection is used to provide detection selectivity [3,6,8,31]. The reversed-phase LC analysis of the total PAH fraction with wavelength programmed fluorescence detection for SRM 1649a is shown in Fig. 15.3. Benzo[*b*]fluoranthene was determined in a second LC–FL analysis of the same total PAH fraction using excitation and emission wavelengths selective for benzo[*b*]fluoranthene instead of the wavelengths used for perylene in the first LC–FL method. A total of 14 PAHs were measured in the LC–FL analysis of the total PAH fraction; however,

$$\lambda_1 = 300/500 \quad \lambda_6 = 252/352$$
$$\lambda_2 = 249/362 \quad \lambda_7 = 285/385$$
$$\lambda_3 = 250/400 \quad \lambda_8 = 263/358$$
$$\lambda_4 = 285/450 \quad \lambda_9 = 406/440$$
$$\lambda_5 = 333/390 \quad \lambda_{10} = 296/405$$
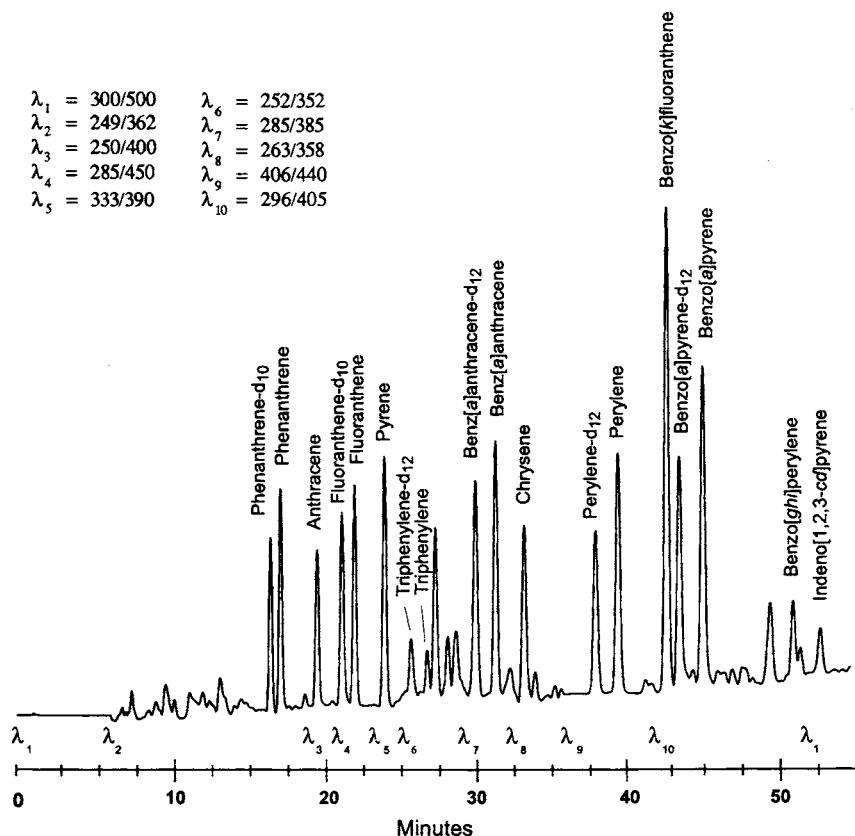
Fig. 15.3. Reversed-phase LC–FL analysis of total PAH fraction from SRM 1649a.

only results for 8 PAHs were considered sufficiently reliable for use in the assignment of the certified values. Even though it was possible to measure triphenylene, benz[a]anthracene, chrysene, benzo[ghi]perylene, and indeno[1,2,3-cd]pyrene in the total PAH fraction, the results for these PAHs were considered as questionable because of possible coelution of minor components with either the analyte of interest or the internal standards. The LC–FL analyses of the four, five, and six aromatic ring isomer fractions isolated from the multidimensional LC procedure were used to overcome these limitations. A detailed discussion of the comparison of the results from the total PAH fraction and the isomer fractions is presented elsewhere [5].

The LC–FL analysis of the four and six aromatic ring fractions are shown in Fig. 15.4A,B. The 278 molecular isomers were determined using the approach described previously by Wise et al. [31]. The LC–FL analysis of the five aromatic ring *cata*-condensed PAH isomers is shown in Fig. 15.4C. Only using this procedure, i.e., normal-phase LC fractionation to isolate isomeric PAH fractions followed by reversed-phase LC analysis of the fractions with fluorescence detection, is it feasible to obtain the necessary sensitivity and selectivity for accurate determination of these 278 molecular weight isomers using LC.
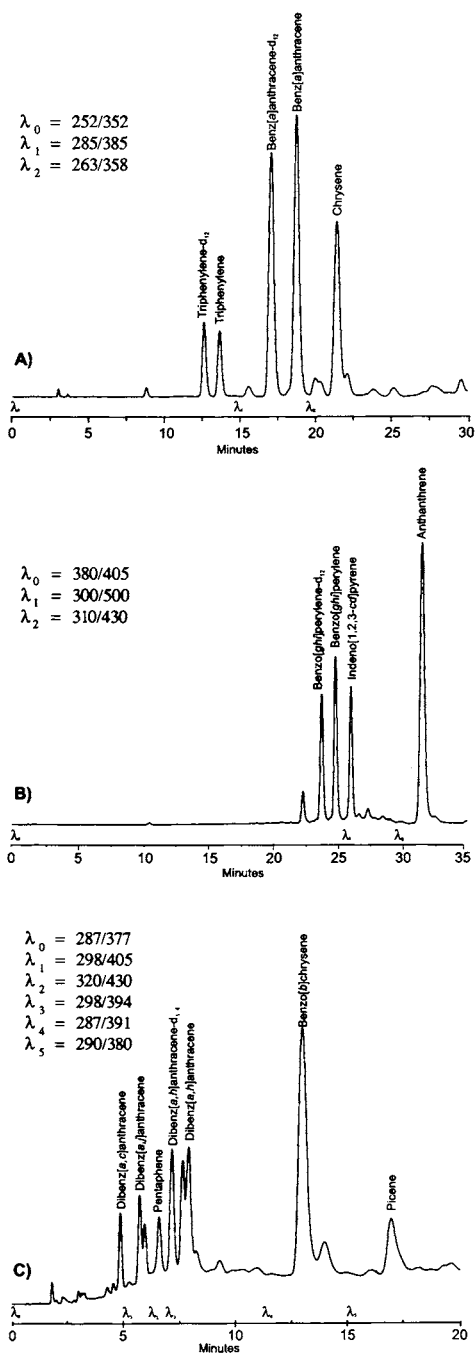
Fig. 15.4. Reversed-phase LC–FL analysis of isomer fractions isolated from SRM 1649a. (A) Four aromatic ring fraction (molecular weight 228), (B) six aromatic ring fraction (molecular weight 276), (C) five aromatic ring fraction (molecular weight 278).

Perdeuterated PAHs are used as internal standards for both the LC–FL and GC/MS methods. In many cases different perdeuterated PAHs are used in the various methods for the quantification of a particular PAH, thereby providing additional independence in the methods. The use of ^{13}C-labeled PAHs as internal standards has also been investigated recently as part of the certification measurements for a mussel tissue SRM [32].

15.4.2 Analytical approach for the determination of PCBs and pesticides

The certification of PCB congeners and chlorinated pesticides in natural matrix SRMs requires the same general approach, using at least two independent analytical techniques, as described above for PAHs. However, in contrast to using both LC and GC for the PAH measurements, GC is the only suitable chromatographic technique at present for the measurement of PCBs and pesticides because of the low levels in environmental matrices and the selectivity and sensitivity of electron capture (ECD) and MS detection used for their measurement. For the first generation of natural matrix SRMs, only a limited number of certified values or only noncertified values were provided for individual PCB congeners and pesticides due to the lack of suitable independence of the analytical techniques. In the early 1990s different analytical techniques were evaluated for the measurement of PCB congeners and chlorinated pesticides to implement more independent analytical techniques and to expand the number of PCB congeners and chlorinated pesticides with certified concentrations in future SRMs. Schantz et al. [33] compared results obtained using different cleanup procedures, three different GC stationary phases, and two different detection methods (ECD and MS) for several existing natural matrix SRMs. This study was the basis for the two independent techniques approach currently used at NIST for providing certified values for individual PCBs and pesticides in natural matrix SRMs. The first SRM issued using this approach for value assignment of PCBs and pesticides was SRM 1945 Organics in Whale Blubber [14]. Basically, the approach consists of different solvent extraction and cleanup procedures followed by analysis using: (1) GC–ECD on two or more stationary phases with different selectivity for PCB congener separations and (2) GC/MS on one or more stationary phases. This approach has been used for all of the SRMs listed in Table 15.4 with certified values for PCBs and pesticides and has been described in detail for SRM 1945 [14], SRM 1941a [9], SRM 1974a [12], and SRM 1649a [34]. In recent certification measurements PFE has also been used as an additional extraction technique based on the study of Schantz et al. [22] establishing the comparability of PFE and Soxhlet extraction for PCBs and pesticides. Recently we have also incorporated the use of ^{13}C-labeled PCB congeners and pesticides as internal standards for quantification in the analysis of mussel tissue materials [32]. The analytical scheme for the recent certification of PCBs and pesticides in SRM 1944 is illustrated in Fig. 15.5. Eight sets of data were used for the value assignment of PCBs and pesticides in SRM 1944. Soxhlet extraction or PFE using two different solvents (dichloromethane and hexane/acetone) were used to extract the PCBs and pesticides from the sediment; the extract was then cleaned up using SPE. For five of the data sets the extracts were separated into two fractions (one containing the PCBs and the second containing the more polar pesticides) using NPLC on an aminopropylsilane column; these two fractions were then analyzed by GC–ECD on two columns of different selectivities, i.e., 5% phenyl methylpolysiloxane and 10% octadecyl (C18) methylpolysiloxane, and GC/MS. In addition to the analyses performed at NIST, SRM 1944 was used in
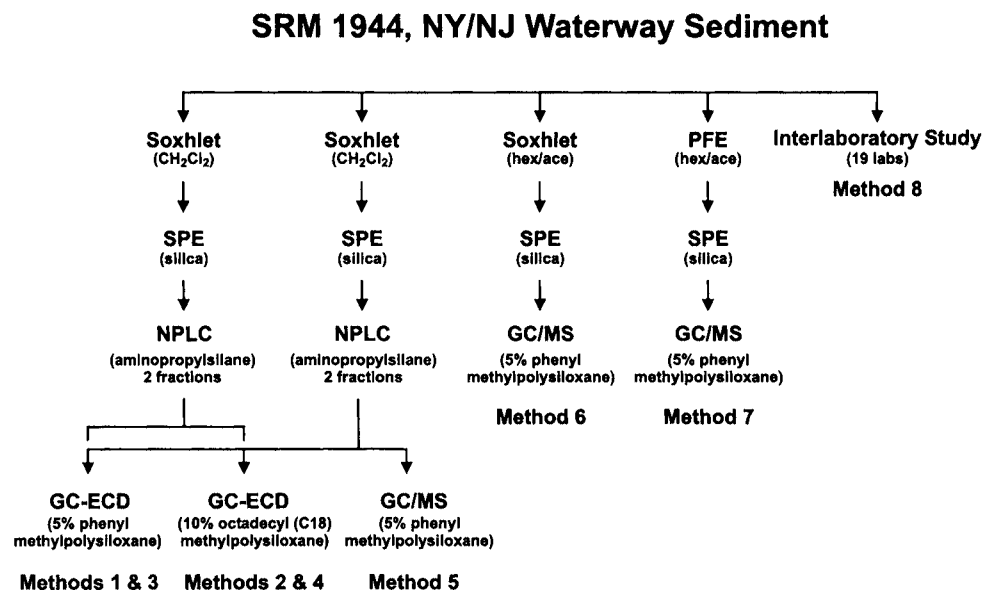
Fig. 15.5. Analytical scheme for the certification of PCBs and pesticides in SRM 1944.

an interlaboratory comparison exercise in 1995 as part of the NIST Intercomparison Exercise Program for Organic Contaminants in the Marine Environment [35]. Results from 19 laboratories that participated in this exercise were used as the eighth data set in the determination of the certified values for PCB congeners and chlorinated pesticides in SRM 1944. The laboratories participating in this exercise used the analytical procedures routinely used in their laboratories to measure PCB congeners and chlorinated pesticides.

15.4.3 Use of other modes of certification

As described above, the primary mode of value assignment at NIST for organic contaminants in natural matrices has been the use of two or more independent analytical methods (mode 2 in Table 15.2). However, as the need for more SRM matrices and/or SRMs with values assigned for different analyte classes increases, NIST will have to rely on the expertise of other laboratories to assist in the value assignment process by providing measurements, thereby maximizing NIST resources. The recent value assignment of concentrations for the seventeen 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxin (PCDD) and dibenzofuran (PCDF) congeners and total tetra-, penta-, hexa-, and hepta-substituted congeners of PCDDs and PCDFs in SRM 1944 and SRM 1649a is an excellent example of the use of results from outside collaborating laboratories. Because NIST did not have the expertise necessary to provide measurements for PCDDs and PCDFs, NIST and Environment Canada coordinated an interlaboratory study among 14 laboratories that routinely measure PCDDs and PCDFs to analyze two existing natural matrix SRMs [36]. The results from this exercise were used to provide reference values (mode 7 in Table 15.2) for these compounds (see discussion later).

We have also used results obtained from an interlaboratory study as an additional set of results in the two or more methods approach (mode 2 in Table 15.2). For example in the recent value assignment for PCBs and pesticides in SRM 1944 described above, the mean of results from 19 laboratories participating in an interlaboratory comparison exercise was used as an additional set of data in the determination of the certified values (see Fig. 15.5). Interlaboratory study results were also included in the value assignment of PAHs, PCBs, and pesticides for the two new mussel tissue materials, SRM 2977 and SRM 2978.

15.5 RECENT SRM ACTIVITIES

Since the previous review in 1993 [17] the NIST SRM activities related to organic contaminants in environmental samples have focused primarily on the development of natural matrix materials. These activities include: (1) updating the certified and reference values on existing materials (i.e., recertification), (2) replacing materials that are no longer available (i.e., renewals), and (3) producing new natural matrix materials. The concentrations of selected PAHs, PCB congeners, and pesticides in the natural matrix SRMs developed in the past six years are summarized in Tables 15.5–15.16. Concentrations for 25 PAHs, most of which are certified values, are summarized for sediment and air particulate SRMs (Table 15.5), mussel tissue SRMs (Table 15.6), and diesel particulate-related SRMs (Table 15.7). Reference values for additional PAHs in these same SRMs are summarized in Tables 15.8–15.10. The concentrations for 29 PCB congeners are summarized for fish oil and whale blubber (Table 15.11), sediment and air particulate SRMs (Table 15.12), and

TABLE 15.5

CERTIFIED AND REFERENCE CONCENTRATIONS OF PAHs IN SEDIMENT AND AIR PARTICULATE SRMs[a]

	SRM 1941a (μg/kg)	SRM 1944 (mg/kg)	SRM 1649a (mg/kg)
Naphthalene	1010 ± 140	1.65 ± 0.31	
Phenanthrene	489 ± 23	5.27 ± 0.22	4.14 ± 0.37
Anthracene	184 ± 14	1.77 ± 0.33	0.432 ± 0.082
Fluoranthene	981 ± 78	8.92 ± 0.32	6.45 ± 0.18
Pyrene	811 ± 24	9.70 ± 0.42	5.29 ± 0.25
Benzo[c]phenathrene	(80 ± 39)	0.76 ± 0.10	(0.46 ± 0.03)
Benz[a]anthracene	427 ± 25	4.72 ± 0.11	2.21 ± 0.073
Chrysene	380 ± 24	4.86 ± 0.10	3.049 ± 0.060
Triphenylene	197 ± 11	1.04 ± 0.27	1.357 ± 0.054
Benzo[b]fluoranthene	740 ± 110	3.87 ± 0.42	6.45 ± 0.64
Benzo[j]fluoranthene	(341 ± 22)	2.09 ± 0.44	(1.5 ± 0.4)
Benzo[k]fluoranthene	361 ± 18	2.30 ± 0.20	1.913 ± 0.031
Benzo[a]fluoranthene	118 ± 11	0.78 ± 0.12	0.409 ± 0.035
Benzo[e]pyrene	553 ± 59	3.28 ± 0.11	3.09 ± 0.19
Benzo[a]pyrene	628 ± 52	4.30 ± 0.13	2.509 ± 0.087
Perylene	452 ± 58	1.17 ± 0.24	0.646 ± 0.075
Anthanthrene	(129 ± 10)	(0.9 ± 0.1)	0.450 ± 0.067
Benzo[ghi]perylene	525 ± 67	2.84 ± 0.10	4.01 ± 0.91
Indeno[1,2,3-cd]pyrene	501 ± 72	2.78 ± 0.10	3.18 ± 0.72
Dibenz[a,j]anthracene	74.3 ± 6.8	0.500 ± 0.044	0.310 ± 0.034
Dibenz[a,c]anthracene	43.1 ± 3.7	0.335 ± 0.013	0.200 ± 0.025
Dibenz[a,h]anthracene	73.9 ± 9.7	0.424 ± 0.069	0.288 ± 0.023
Pentaphene	42 ± 12	0.288 ± 0.026	0.151 ± 0.035
Benzo[b]chrysene	99 ± 20	0.63 ± 0.10	0.315 ± 0.013
Picene	80.0 ± 9.0	0.518 ± 0.093	0.426 ± 0.022

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

mussel tissue SRMs (Table 15.13). Certified concentrations for 13 chlorinated pesticides in these same materials are provided in Tables 15.14–15.16.

The certified values in Tables 15.5–15.7 and Tables 15.11–15.16 are typically the mean of the means from two or more analytical methods, weighted as described in Paule and Mandel [37]. The uncertainty associated with each certified value is computed according to the CIPM approach as described in the ISO Guide [38] and is an expanded uncertainty at the 95% level of confidence, which includes random sources of uncertainty within each analytical method as well as uncertainty due to drying studies (when applicable). This expanded uncertainty defines a range of values within which the true value is believed to lie, at a level of confidence of approximately 95%. The reference values in Tables 15.8–15.10, as well as those included in Tables 15.5–15.7 and Tables 15.11–15.16, are typically the equally-weighted mean of the means from two or more analytical methods or the mean from one analytical technique. The uncertainty in the reference value defines a range of values that is intended to function as an interval that contains the true value at a level of

TABLE 15.6

CERTIFIED AND REFERENCE CONCENTRATIONS OF PAHs IN MUSSEL TISSUE SRMs[a]

	SRM 1974a (μg/kg wet-basis)	SRM 2974 (μg/kg dry-basis)	SRM 2978 (μg/kg dry-basis)	SRM 2977 (μg/kg dry-basis)
Naphthalene	2.68 ± 0.50	(9.63 ± 0.61)	(31 ± 6)	19 ± 5
Phenanthrene	2.53 ± 0.28	22.2 ± 2.5	(74 ± 7)	35.1 ± 3.8
Anthracene	0.69 ± 0.20	6.1 ± 1.7	(5.4 ± 2.2)	(8 ± 4)
Fluoranthene	18.6 ± 1.0	163.7 ± 10.3	166 ± 12	38.7 ± 1.0
Pyrene	17.26 ± 0.74	151.6 ± 8.0	256 ± 21	78.9 ± 3.5
Benz[a]anthracene	3.71 ± 0.54	32.5 ± 4.8	(25 ± 2)	20.3 ± 0.8
Chrysene	5.04 ± 0.26	44.2 ± 2.7	(59 ± 10)	(49 ± 2)
Triphenylene	5.77 ± 0.67	50.7 ± 6.1	(63 ± 9)	(39 ± 1)
Benzo[a]fluoranthene	(0.45 ± 0.22)	(4.0 ± 1.9)		
Benzo[b]fluoranthene	5.28 ± 0.42	46.4 ± 4.0	(58 ± 15)	11.0 ± 0.3
Benzo[j]fluoranthene	(2.33 ± 0.20)	(20.5 ± 1.8)	(23 ± 2)	(4.6 ± 0.2)
Benzo[k]fluoranthene	2.30 ± 0.10	20.2 ± 1.0	24.1 ± 3.4	(4 ± 1)
Benzo[e]pyrene	9.56 ± 0.21	84.0 ± 3.2	89.3 ± 6.3	13.1 ± 1.1
Benzo[a]pyrene	1.780 ± 0.073	15.63 ± 0.80	(7 ± 3)	8.35 ± 0.72
Perylene	0.874 ± 0.030	7.68 ± 0.35	4.09 ± 0.32	3.50 ± 0.76
Benzo[ghi]perylene	2.50 ± 0.25	22.0 ± 2.3	19.7 ± 4.4	9.53 ± 0.43
Indeno[1,2,3-cd]pyrene	1.62 ± 0.32	14.2 ± 2.8	12.2 ± 2.9	4.84 ± 0.81
Anthanthrene	(0.131 ± 0.036)	(1.15 ± 0.31)		
Dibenz[a,j]anthracene	(0.142 ± 0.010)	(1.247 ± 0.084)		
Dibenz[a, c + a, h]anthracene	(0.342 ± 0.022)	(3.00 ± 0.22)	(3.5 ± 0.5)	(2.0 ± 0.2)
Dibenz[a,h]anthracene				1.41 ± 0.19
Benzo[b]chrysene	(0.182 ± 0.016)	(1.60 ± 0.16)	(2.1 ± 0.4)	1.07 ± 0.15
Picene			(4.5 ± 0.5)	2.29 ± 0.27

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM or the Report of Investigation for the RM.

confidence of 95%. This uncertainty includes sources of uncertainty within each analytical method, among methods, and from the drying study (when applicable) but may only represent a measure of the precision of the measurement method(s). The method for assigning the certified and reference values and the associated uncertainties for each SRM may differ slightly; the specific details are contained in each SRM Certificate of Analysis.

15.5.1 Updated certification

At NIST when a natural matrix SRM is developed, a sufficient quantity is typically produced to last for 7–8 years. If the supply of the SRM is depleted after this time, a new batch of a similar material is prepared and issued as a renewal SRM (see discussion below). However, for several of the SRMs issued during the past 20 years, sufficient material was still available after 8–10 years to justify reanalyzing the material (i.e., a

TABLE 15.7

CERTIFIED AND REFERENCE CONCENTRATIONS OF PAHs IN DIESEL PARTICULATE SRMs[a]

	SRM 1650a (mg/kg)	SRM 2975 (mg/kg)	SRM 1975 (mg/kg)
Phenanthrene	68.4 ± 8.5	17.0 ± 2.8	8.08 ± 0.32
Anthracene	(1.5 ± 0.6)	(0.038 ± 0.008)	
Fluoranthene	49.9 ± 2.7	26.6 ± 5.1	13.74 ± 0.66
Pyrene	47.5 ± 2.7	0.90 ± 0.24	(0.40 ± 0.14)
Benzo[c]phenathrene	2.75 ± 0.63	(1.0 ± 0.4)	(0.48 ± 0.09)
Benz[a]anthracene	6.33 ± 0.77	0.317 ± 0.066	0.0804 ± 0.0055
Chrysene	14.5 ± 0.8	4.56 ± 0.16	1.90 ± 0.16
Triphenylene	11.5 ± 1.6	5.22 ± 0.20	2.39 ± 0.17
Benzo[b]fluoranthene	8.81 ± 0.60	(11.5 ± 3.6)	3.21 ± 0.12
Benzo[j]fluoranthene	3.52 ± 0.40	0.82 ± 0.11	
Benzo[k]fluoranthene	2.64 ± 0.31	0.678 ± 0.077	0.1473 ± 0.0082
Benzo[a]fluoranthene	0.44 ± 0.08	(0.061 ± 0.016)	
Benzo[e]pyrene	7.44 ± 0.53	1.11 ± 0.10	0.266 ± 0.024
Benzo[a]pyrene	1.33 ± 0.35	0.0522 ± 0.0053	
Perylene	0.16 ± 0.04	0.054 ± 0.009	
Benzo[ghi]perylene	6.50 ± 0.94	0.498 ± 0.044	(0.04 ± 0.02)
Indeno[1,2,3-cd]pyrene	5.62 ± 0.53	(1.38 ± 0.19)	(0.13 ± 0.02)
Dibenz[a,j]anthracene	0.52 ± 0.10	(0.37 ± 0.07)	
Dibenz[a,c]anthracene	0.500 ± 0.063		
Dibenz[a,h]anthracene	(0.89 ± 0.21)		(0.08 ± 0.02)
Pentaphene	(0.24 ± 0.11)	(0.038 ± 0.007)	
Benzo[b]chrysene	0.316 ± 0.038	(0.08 ± 0.03)	(0.12 ± 0.06)
Picene	0.620 ± 0.081	(1.0 ± 0.2)	

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

new certification) to provide new certified and reference values for the original analytes measured as well as for additional analytes of interest. Environmental matrix SRMs need to be updated or recertified as analytical measurement capabilities improve and/or as the need for value assignment for more analytes increases. The goals of the recertification efforts are generally: (1) to provide an increased number of certified concentrations for PAHs, PCBs, pesticides, and other analytes, (2) to reduce the uncertainties associated with the certified concentrations, and (3) to assess the stability of previously measured analytes since the original certification measurements. The natural matrix SRMs recently recertified are listed in Table 15.17 and include SRM 1588a, SRM 1649a, SRM 1650a, and SRM 1939a. The number of certified and reference values for PAHs, PCBs, and pesticides determined in the original certification are compared to those determined in the recertification.

15.5.1.1 Recertification of SRM 1649a Urban Dust

An excellent example of the need to update and recertify an existing SRM is SRM 1649,

TABLE 15.8

REFERENCE CONCENTRATIONS OF ADDITIONAL PAHs IN SEDIMENT AND AIR PARTICULATE SRMs[a]

	SRM 1941a (µg/kg)	SRM 1944 (mg/kg)	SRM 1649a (mg/kg)
1-Methylnaphthalene		0.52 ± 0.03	
2-Methylnaphthalene		0.95 ± 0.05	
Biphenyl	175 ± 18	0.32 ± 0.07	
Acenaphthylene	37 ± 14		
Acenaphthene	41 ± 10	0.57 ± 0.03	
Fluorene	97.3 ± 8.6[b]	0.85 ± 0.03	0.23 ± 0.05
Dibenzothiophene	70.0 ± 9.4	0.62 ± 0.01	0.18 ± 0.01
1-Methylphenanthrene	101 ± 27	1.7 ± 0.1	0.37 ± 0.04
2-Methylphenanthrene	158 ± 32	1.90 ± 0.06	0.73 ± 0.12
3-Methylphenanthrene	97 ± 32	2.1 ± 0.1	0.50 ± 0.05
4+9-Methylphenanthrene		1.6 ± 0.2	0.34 ± 0.01
2-Methylanthracene		0.58 ± 0.04	
4H-Cyclopenta[*def*]-phenanthrene	92 ± 15		0.32 ± 0.06
Benzo[*ghi*]fluoranthene	97.9 ± 3.1		0.88 ± 0.02
Indeno[1,2,3-*cd*]fluoranthene	20.0 ± 2.3		0.080 ± 0.004

[a] For detailed description of the uncertainties associated with the reference values, see the Certificate of Analysis for each SRM.

[b] Certified value.

Urban Dust/Organics. SRM 1649, the first particle-based natural matrix material developed by NIST for organic contaminants, was issued in 1982 with certified concentration values for only five PAHs and noncertified concentrations for nine additional PAHs [3,4]. The certified values for the five PAHs were based on the combined results of analyses by two analytical techniques, GC–FID and reversed-phase LC–FL. The values reported for the remaining nine PAHs were based on results from only one analytical technique (i.e., either GC–FID or LC–FL). The relative uncertainties associated with the certified values ranged from a low of 7% (fluoranthene) to a high of 24% (benzo[*ghi*]perylene) with the remaining three PAHs at 12% to 17%, which were considered as adequate for the first particle-based matrix SRM.

Since SRM 1649 was first issued in 1982, the state-of-art for the measurement of PAHs has improved significantly. During this time NIST developed and implemented improved analytical methods for the measurement and certification of a significantly greater number of PAHs in environmental matrix SRMs. Because there was still a considerable supply of SRM 1649 available even after 17 years, recertification of SRM 1649 was undertaken. The recertified air particulate material was reissued in 1998 as SRM 1649a, Urban Dust, using the analytical approach described in Section 15.4.1. The recertification of SRM 1649a for PAHs has been described in detail elsewhere [5]. Results from as many as five different analytical techniques were combined to provide certified concentrations for 22 PAHs and reference concentrations for an additional 23 PAHs (see Fig. 15.1). The relative uncertainties associated with the certified concentrations in SRM 1649a ranged from a low of

TABLE 15.9

REFERENCE CONCENTRATIONS OF ADDITIONAL PAHs IN TISSUE SRMs[a]

	SRM 1974a (μg/kg wet-basis)	SRM 2974 (μg/kg dry-basis)	SRM 2978 (μg/kg dry-basis)	SRM 2977 (μg/kg dry-basis)
1-Methylnaphthalene	0.61 ± 0.20	3.47 ± 0.85	21 ± 5	16 ± 5
2-Methylnaphthalene	1.16 ± 0.17	6.48 ± 0.85	23 ± 4	18 ± 5
Biphenyl	0.582 ± 0.038	4.68 ± 0.56	8 ± 1	6.8 ± 0.6
Acenaphthylene	0.598 ± 0.043	4.60 ± 0.88	4 ± 1	4.2 ± 0.4
Acenaphthene	0.359 ± 0.038	2.74 ± 0.52	6 ± 2	
Fluorene	0.65 ± 0.10	4.69 ± 0.34	7 ± 1	10.2 ± 0.4
1-Methylphenanthrene	1.20 ± 0.55	10.5 ± 4.8		44 ± 2
2-Methylphenanthrene	2.34 ± 0.92	20.6 ± 8.0		43 ± 1
3-Methylphenanthrene	1.5 ± 1.1	13.5 ± 9.7		44.2 ± 0.4
4 + 9-Methylphenanthrene	1.7 ± 1.0	14.7 ± 9.2		36 ± 2
Benzo[ghi]fluoranthene	3.22 ± 0.62			

[a] For detailed description of the uncertainties associated with the reference values, see the Certificate of Analysis for each SRM.

2% to a high of 24% with most of the uncertainties in the 5% to 10% range (see Table 15.18).

The recertification of SRM 1649 provided an excellent opportunity to evaluate the stability of the air particulate material after a period of about 17 years. The results of the original certification (SRM 1649) are compared with the results of the recertification (SRM 1649a) in Table 15.18 for the 14 PAHs measured in the original SRM 1649 (five

TABLE 15.10

REFERENCE CONCENTRATIONS OF ADDITIONAL PAHs IN DIESEL PARTICULATE SRMs[a]

	SRM 1650a (mg/kg)	SRM 2975 (mg/kg)	SRM 1975 (mg/kg)
1-Methylnaphthalene			0.39 ± 0.04
2-Methylnaphthalene			0.69 ± 0.07
Biphenyl			0.24 ± 0.01
Fluorene			0.110 ± 0.007
1-Methylphenanthrene	34 ± 7	0.89 ± 0.11	0.50 ± 0.05
2-Methylphenanthrene	70 ± 4	2.0 ± 0.2	1.7 ± 0.3
3-Methylphenanthrene	57 ± 8	1.0 ± 0.2	0.9 ± 0.2
4+9-Methylphenanthrene	33 ± 9	0.44 ± 0.09	0.25 ± 0.04
Benzo[ghi]fluoranthene	12.1 ± 0.3	10.2 ± 0.5	4.3 ± 0.5
Indeno[1,2,3-cd]-fluoranthene		1.1 ± 0.2	
Benzo[c]chrysene	0.60 ± 0.09		

[a] For detailed description of the uncertainties associated with the reference values, see the Certificate of Analysis for each SRM.

TABLE 15.11

CERTIFIED CONCENTRATIONS FOR SELECTED PCB CONGENERS IN FISH OIL AND WHALE BLUB-BER SRMs[a]

PCB	No.	(PCB Name)[b]	SRM 1588a (µg/kg wet-basis)	SRM 1945 (µg/kg wet-basis)
PCB	18	(2,2',5-Trichlorobiphenyl)	(8.1 ± 2.2)	4.48 ± 0.88
PCB	28	(2,4,4'-Trichlorobiphenyl)	28.32 ± 0.55	(14.1 ± 1.4)
PCB	31	(2,4',5-Trichlorobiphenyl)	8.33 ± 0.28	(3.12 ± 0.69)
PCB	44	(2,2',3,5'-Tetrachlorobiphenyl)	35.1 ± 1.4	12.2 ± 1.4
PCB	49	(2,2',4,5'-Tetrachlorobiphenyl)	29.90 ± 0.84	20.8 ± 2.8
PCB	52	(2,2',5,5'-Tetrachlorobiphenyl)	83.3 ± 2.3	43.6 ± 2.5
PCB	66	(2,3',4,4'-Tetrachlorobiphenyl)	54.7 ± 1.5	23.6 ± 1.6
PCB	95	(2,2',3,5',6-Pentachlorobiphenyl)	36.5 ± 1.1	33.8 ± 1.7
PCB	87	(2,2',3,4,5'-Pentachlorobiphenyl)	56.3 ± 1.1	16.7 ± 1.4
PCB	99	(2,2',4,4',5-Pentachlorobiphenyl)		45.4 ± 5.4
PCB	101	(2,2',4,5,5'-Pentachlorobiphenyl)	126.5 ± 4.3	65.2 ± 5.6
	90	(2,2',3,4',5-Pentachlorobiphenyl)		
PCB	105	(2,3,3',4,4'-Pentachlorobiphenyl)	60.2 ± 2.3	30.1 ± 2.3
PCB	110	(2,3,3',4',6-Pentachlorobiphenyl)	76.0 ± 2.0	23.3 ± 4.0
PCB	118	(2,3',4,4',5-Pentachlorobiphenyl)	176.3 ± 3.8	74.6 ± 5.1
PCB	128	(2,2',3,3',4,4'-Hexachlorobiphenyl)	47.0 ± 2.4	23.7 ± 1.7
PCB	138	(2,2',3,4,4',5'-Hexachlorobiphenyl)	263.5 ± 9.1	131.5 ± 7.4
	163	(2,3,3',4',5,6-Hexachlorobiphenyl)		
	164	(2,3,3',4',5',6-Hexachlorobiphenyl)		
PCB	149	(2,2',3,4',5',6-Hexachlorobiphenyl)	105.7 ± 3.6	106.7 ± 5.3
PCB	151	(2,2',3,5,5',6-Hexachlorobiphenyl)	54.8 ± 2.1	28.7 ± 5.2
PCB	153	(2,2',4,4',5,5'-Hexachlorobiphenyl)	273.8 ± 7.7	213 ± 19
PCB	156	(2,3,3',4,4',5-Hexachlorobiphenyl)	27.3 ± 1.8	10.3 ± 1.1
PCB	170	(2,2',3,3',4,4',5-Heptachlorobiphenyl)	46.5 ± 1.1	40.6 ± 2.6
	190	(2,3,3',4,4',5,6-Heptachlorobiphenyl)		
PCB	180	(2,2',3,4,4',5,5'-Heptachlorobiphenyl)	105.0 ± 5.2	106.6 ± 8.4
PCB	183	(2,2',3,4,4',5',6-Heptachlorobiphenyl)	31.21 ± 0.62	36.6 ± 4.1
PCB	187	(2,2',3,4',5,5',6-Heptachlorobiphenyl)	35.23 ± 0.83	105.1 ± 9.1
	159	(2,3,3',4,5,5'-Hexachlorobiphenyl)		
	182	(2,2',3',4,4',5,6-Heptachlorobiphenyl)		
PCB	194	(2,2',3,3',4,4',5,5'-Octachlorobiphenyl)	15.37 ± 0.61	39.6 ± 2.5
PCB	195	(2,2',3,3',4,4',5,6-Octachlorbiphenyl)	(4.6 ± 0.6)	17.7 ± 4.3
PCB	201	(2,2',3,3',4,5',6,6'-Octachlorbiphenyl)	12.18 ± 0.46	16.96 ± 0.89
PCB	206	(2,2',3,3',4,4',5,5',6-Nonachlorobiphenyl)	(3.4 ± 1.6)	31.1 ± 2.7
PCB	209	Decachlorobiphenyl	(3.5 ± 1.0)	10.6 ± 1.1

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

[b] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [54] and later revised by Schulte and Malisch [55] to conform with IUPAC rules; for the specific congeners mentioned in these SRMs, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. When two or more congeners are known to coelute under the conditions used, the PCB congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.

TABLE 15.12

CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED PCBs IN SEDIMENT AND AIR PARTICULATE SRMs[a]

PCB	No.[b]	SRM 1941a (μg/kg)	SRM 1944 (μg/kg)	SRM 1939a (μg/kg)	SRM 1649a (μg/kg)
PCB	8	(1.39 ± 0.19)	22.3 ± 2.3		12.28 ± 0.29
PCB	18	(1.15 ± 0.16)	51.0 ± 2.6	(3210 ± 940)	20.44 ± 0.84
PCB	28	(9.8 ± 3.7)	80.8 ± 2.7	(2461 ± 78)	18.5 ± 1.2
PCB	31	(6.2 ± 2.4)	78.7 ± 1.6	(6440 ± 490)	17.3 ± 1.4
PCB	44	4.80 ± 0.62	60.2 ± 2.0	1131 ± 74	15.4 ± 1.6
PCB	49	9.5 ± 2.1	53.0 ± 1.7	3740 ± 280	12.2 ± 1.5
PCB	52	6.89 ± 0.56	79.4 ± 2.0	4320 ± 130	24.65 ± 0.97
PCB	66	6.8 ± 1.4	71.9 ± 4.3	840 ± 130	65 ± 12
PCB	95	7.5 ± 1.1	65.0 ± 8.9	(1210 ± 420)	51.6 ± 4.2
PCB	87	6.70 ± 0.37	29.9 ± 4.3		10.65 ± 0.62
PCB	99	4.17 ± 0.51	37.5 ± 2.4	380 ± 96	9.58 ± 0.69
PCB	101/90	11.0 ± 1.6	73.4 ± 2.5		52.9 ± 1.0
PCB	105	3.65 ± 0.27	24.5 ± 1.1	201 ± 28	8.63 ± 0.80
PCB	110	9.47 ± 0.85	63.5 ± 4.7	1068 ± 70	26.6 ± 1.6
PCB	118	10.0 ± 1.1	58.0 ± 4.3	423 ± 88	25.7 ± 1.5
PCB	128	1.87 ± 0.32	8.47 ± 0.28	91.2 ± 8.4	6.35 ± 0.69
PCB	138/163/164	13.38 ± 0.97	62.1 ± 3.0	258.1 ± 6.9	69.7 ± 7.5
PCB	149	9.2 ± 1.1	49.7 ± 1.2	427 ± 47	75.7 ± 1.3
PCB	151	(2.62 ± 0.22)	16.93 ± 0.36	192.1 ± 2.6	34.3 ± 3.9
PCB	153	17.6 ± 1.9	74.0 ± 2.9	297 ± 19	82.5 ± 8.0
PCB	156	0.93 ± 0.14	6.52 ± 0.66	37.0 ± 6.6	16.25 ± 0.77
PCB	170/190	3.00 ± 0.46	22.6 ± 1.4	107 ± 17	30.8 ± 2.2
PCB	180	5.83 ± 0.58	44.3 ± 1.2	140.3 ± 6.1	78.7 ± 8.2
PCB	183	(1.63 ± 0.15)	12.19 ± 0.57	47.3 ± 2.3	20.34 ± 0.95
PCB	187/159/182	(7.0 ± 2.6)	25.1 ± 1.0	156.4 ± 2.6	40.1 ± 2.5
PCB	194	1.78 ± 0.23	11.2 ± 1.4	35.5 ± 4.1	28.9 ± 3.6
PCB	206	3.67 ± 0.87	9.21 ± 0.51	29.7 ± 5.6	20.6 ± 4.6
PCB	209	8.34 ± 0.49	6.81 ± 0.33		8.04 ± 0.77

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

[b] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [54] and later revised by Schulte and Malisch [55] to conform with IUPAC rules; for the specific congeners mentioned in these SRMs, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. When two or more congeners are known to coelute under the conditions used, the PCB congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.

certified and nine noncertifed). Table 15.18 also includes the percent difference between the SRM 1649 and 1649a results and an indication (designated with an X in the final column) of whether the uncertainties on the 'new' and 'old' certified values overlap (or in the case of the noncertifed values whether the measurement uncertainties overlap). As shown in Table 15.18, all the values for the original five PAHs with certified values have

TABLE 15.13

CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED PCBs IN MUSSEL TISSUE SRMs[a]

PCB	No.[b]	SRM 1974a (μg/kg wet-basis)	SRM 2974 (μg/kg dry-basis)	SRM 2978 (μg/kg dry-basis)	SRM 2977 (μg/kg dry-basis)
PCB	8				2.10 ± 0.15
PCB	18	(3.7 ± 1.2)	(26.8 ± 3.3)	(7 ± 2)	2.65 ± 0.30
PCB	28	(9.0 ± 1.7)	(79 ± 15)	7.91 ± 0.90	5.37 ± 0.44
PCB	31	(8.6 ± 2.4)	(76 ± 21)	21.40 ± 0.43	3.92 ± 0.24
PCB	44	8.28 ± 0.84	72.7 ± 7.7	11.80 ± 0.64	3.25 ± 0.63
PCB	49	10.12 ± 0.59	88.8 ± 5.7	16.84 ± 0.86	
PCB	52	13.1 ± 1.3	115 ± 12	17.7 ± 2.8	8.37 ± 0.54
PCB	66	11.54 ± 0.50	101.4 ± 5.4	18.4 ± 1.5	3.64 ± 0.32
PCB	95	9.5 ± 1.9	83 ± 17	20.8 ± 2.1	5.39 ± 0.59
PCB	87	(6.1 ± 1.6)	(54 ± 14)	10.20 ± 0.29	2.15 ± 0.08
PCB	99	8.08 ± 0.46	70.9 ± 4.5	18.84 ± 0.44	1.59 ± 0.20
PCB	101/90	14.6 ± 1.1	128 ± 10	35.9 ± 1.6	11.2 ± 1.2
PCB	105	6.04 ± 0.39	53.0 ± 3.8	10.85 ± 0.45	3.76 ± 0.49
PCB	110	14.5 ± 1.0	127.3 ± 9.4	35.34 ± 0.71	4.03 ± 0.20
PCB	118	14.90 ± 0.40	130.8 ± 5.3	35.1 ± 1.0	10.5 ± 1.0
PCB	128	2.50 ± 0.39	22.0 ± 3.5	5.25 ± 0.17	2.49 ± 0.28
PCB	138/163/164	15.2 ± 1.1	134 ± 10	35.7 ± 1.5	16.6 ± 1.6
PCB	149	9.98 ± 0.27	87.6 ± 3.5	34.73 ± 0.69	9.23 ± 0.12
PCB	151	2.91 ± 0.40	25.6 ± 3.6	10.92 ± 0.25	3.07 ± 0.18
PCB	153	16.54 ± 0.86	145.2 ± 8.8	56.9 ± 3.5	14.1 ± 1.0
PCB	156	0.85 ± 0.11	7.4 ± 1.0	1.97 ± 0.11	0.96 ± 0.08
PCB	170/190	0.63 ± 0.12	5.5 ± 1.1	(2.4 ± 0.6)	2.95 ± 0.23
PCB	180	1.95 ± 0.43	17.1 ± 3.8	7.81 ± 0.63	6.79 ± 0.67
PCB	183	1.82 ± 0.27	16.0 ± 2.4	5.25 ± 0.15	1.33 ± 0.10
PCB	187/159/182	3.87 ± 0.27	34.0 ± 2.5	16.7 ± 1.3	4.76 ± 0.38

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM or the Report of Investigation for the RM.

[b] PCB congeners are numbered according to the scheme proposed by Ballschmiter and Zell [54] and later revised by Schulte and Malisch [55] to conform with IUPAC rules; for the specific congeners mentioned in these SRMs, the Ballschmiter-Zell numbers correspond to those of Schulte and Malisch. When two or more congeners are known to coelute under the conditions used, the PCB congener listed first is the major component and the additional congeners may be present as minor components. The quantitative results are based on the response of the congener listed first.

decreased from 4% to 15%; the remaining noncertified values have also decreased from 4% to 30% with the exception of benzo[*b*]fluoranthene which increased by 4%. However, the decreases greater than 15% can usually be attributed to improvements in the analytical methodology for these specific PAHs. For example, the original LC–FL measurements of dibenz[*a,h*]anthracene were made with no cleanup of the extract, whereas the recent measurements were performed on the normal-phase LC-isolated 278 molecular weight isomer fraction, which would produce a more reliable result. In the case of the original

TABLE 15.14

CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED CHLORINATED PESTICIDES IN
FISH OIL AND WHALE BLUBBER SRMs[a]

	SRM 1588a (μg/kg wet-basis)	SRM 1945 (μg/kg wet-basis)
Hexachlorobenzene	157.8 ± 5.0	32.9 ± 1.7
α-HCH	85.3 ± 3.4	16.2 ± 3.4
β-HCH		(8.0 ± 1.4)
γ-HCH	24.9 ± 1.7	
cis-Chlordane (α-Chlordane)	167.0 ± 5.0	46.9 ± 2.8
cis-Nonachlor	94.8 ± 2.8	48.7 ± 7.6
trans-Nonachlor	214.6 ± 7.9	231 ± 11
Dieldrin	155.9 ± 4.5	(37.5 ± 3.9)
Oxychlordane		19.8 ± 1.9
2,4'-DDE	22.0 ± 1.0	12.28 ± 0.87
4,4'-DDE	651 ± 11	445 ± 37
2,4'-DDD	36.3 ± 1.5	18.1 ± 2.8
4,4'-DDD	254 ± 11	133 ± 10
2,4'-DDT	156.0 ± 4.4	106 ± 14
4,4'-DDT	524 ± 12	245 ± 15

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

triphenylene measurements, the concentration was determined without the addition of an internal standard, whereas the recent measurements were quantified based on using triphenylene-d_{12} as the internal standard, which had been added prior to extraction and cleanup and therefore should mimic the behavior of triphenylene and provide a more reliable result. In the original Certificate of Analysis, results were reported for pyrene and perylene from two analytical techniques (GC–FID and LC–FL); however, the two results were not in agreement (hence the designation as noncertified, with the LC–FL result lower by 15% and 29%, respectively). Subsequent analyses of SRM 1649 and the comparison of results of LC and GC/MS analyses on other environmental matrix SRMs performed shortly thereafter [39] indicated that the original GC–FID values for pyrene and perylene were overestimated (probably due to coelution of minor compounds); therefore, the LC–FL results were considered as more accurate. Thus it appears that 4–15% is a realistic value for the decrease in the measured concentrations of SRM 1649 between the original certification and the recertification analyses.

This decrease in the concentrations may indicate that the air particulate material has changed during the past 17 years or perhaps the decrease is just an artifact of the improved measurements. The air particulate material has been stored at room temperature in amber glass bottles since the original measurements. The decrease is actually 1% more because the original measurements were reported on an as-received basis whereas the new certified values are reported on a dry-mass basis. Even though the five original certified values have decreased, the new certified values are, with the exception of benz[a]anthracene, still

TABLE 15.15

CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED CHLORINATED PESTICIDES IN SEDIMENT AND AIR PARTICULATE SRMs[a]

	SRM 1941a (μg/kg)	SRM 1944 (μg/kg)	SRM 1939a (μg/kg)	SRM 1649a (μg/kg)
Hexachlorobenzene	70 ± 25	6.03 ± 0.35		16.3 ± 1.8
α-HCH		(2.0 ± 0.3)		
cis-Chlordane (α-Chlordane)	2.33 ± 0.56	16.51 ± 0.83	4.8 ± 1.3	34.88 ± 0.42
trans-Chlordane (γ-Chlordane)		(8 ± 2)		40.3 ± 2.8
cis-Nonachlor		(3.7 ± 0.7)		
trans-Nonachlor	1.26 ± 0.13	8.20 ± 0.51		27.6 ± 1.6
Dieldrin	(1.26 ± 0.37)			
Oxychlordane	(2.59 ± 0.19)			
2,4'-DDE	0.73 ± 0.11	(19 ± 3)		5.79 ± 0.85
4,4'-DDE	6.59 ± 0.56	(86 ± 12)		40.4 ± 1.7
2,4'-DDD		(38 ± 8)		
4,4'-DDD	5.06 ± 0.58	(108 ± 16)	5.50 ± 0.97	34.01 ± 0.48
4,4'-DDT	(1.25 ± 0.10)	119 ± 11	2.72 ± 0.42	212 ± 15

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

within the uncertainties of the original certified values. When the uncertainties of the new certified values and the uncertainties of the old certified values (or the measurement uncertainties of the noncertified values) are considered, 9 of the 14 concentration values overlap. The fact that all of the values decrease suggests that the material has changed. However, all of the analytical methods used in the recertification are more selective relative to cleanup and isolation of the PAH fraction and they used more selective detection in the GC methods (i.e., MS vs. FID), which would tend to produce lower values. Thus, it is difficult to determine definitively whether the material has been stable with respect to the PAH concentrations over the past 17 years.

In addition to the certified and reference values for 44 PAHs, the updated Certificate of Analysis [40] for SRM 1649a also provides certified values for 35 polychlorinated biphenyl congeners and 8 chlorinated pesticides (see Tables 15.12 and 15.15) [34]. Reference values are provided for 32 inorganic constituents, mutagenic activity [41], particle-size characteristics, total organic carbon, total extractable material, and carbon composition. The carbon composition values include the results from both NIST and other laboratories using various techniques for total carbon, insoluble carbon, organic carbon, elemental carbon, pyrolyzed carbon, and carbonate carbon. Reference values are also provided for the 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners (17 individual congeners) and total tetra-, penta-, hexa-, and hepta-congeners of polychlorinated dibenzo-*p*-dioxin and dibenzofuran as determined from an interlaboratory comparison exercise among 14 laboratories (see Table 15.19 and discussion below). With a total of over 160 certified and reference values for constituents or properties, SRM 1649a is currently the most extensively characterized natural environmental matrix SRM available from NIST.

TABLE 15.16

CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED CHLORINATED PESTICIDES IN MUSSEL TISSUE SRMs[a]

	SRM 1974a (μg/kg wet-basis)	SRM 2974 (μg/kg dry-basis)	SRM 2978 (μg/kg dry-basis)	SRM 2977 (μg/kg dry-basis)
cis-Chlordane (α-Chlordane)	1.96 ± 0.32	17.2 ± 2.9	15.56 ± 0.83	1.42 ± 0.13
trans-Chlordane (γ-Chlordane)	1.89 ± 0.19	16.6 ± 1.8	11.38 ± 0.56	
cis-Nonachlor	0.78 ± 0.10	6.84 ± 0.92	8.23 ± 0.56	
trans-Nonachlor	2.05 ± 0.41	18.0 ± 3.6	11.5 ± 1.0	1.43 ± 0.10
Dieldrin	(0.70 ± 0.15)	(6.2 ± 1.3)	6.30 ± 0.67	6.04 ± 0.52
Oxychlordane			(2.13 ± 0.27)	
2,4′-DDE	(0.599 ± 0.031)	(5.26 ± 0.31)	4.41 ± 0.56	
4,4′-DDE	5.84 ± 0.63	51.2 ± 5.7	37.5 ± 1.5	12.5 ± 1.6
2,4′-DDD	(1.56 ± 0.32)	(13.7 ± 2.8)	10.5 ± 1.0	3.32 ± 0.29
4,4′-DDD	4.90 ± 0.72	43.0 ± 6.4	38.8 ± 2.3	4.30 ± 0.38
2,4′-DDT	(0.96 ± 0.21)	(8.5 ± 1.9)	9.2 ± 1.6	
4,4′-DDT	0.455 ± 0.067	3.91 ± 0.60	3.84 ± 0.28	1.28 ± 0.18

[a] All concentrations are certified values except those in parentheses, which are reference values. For detailed description of the uncertainties associated with the values, see the Certificate of Analysis for each SRM.

15.5.1.2 Recertification of SRM 1650a Diesel Particulate Matter

Another of the first natural matrix SRMs developed by NIST is SRM 1650, Diesel Particulate Matter, which was issued in 1985 with certified concentrations for five

TABLE 15.17

RECERTIFICATIONS AND RENEWALS OF PREVIOUS ENVIRONMENTAL MATRIX SRMs

	Original certification[a]			New Certification[a]		
	PAHs	PCBs	Pesticides	PAHs	PCBs	Pesticides
Recertifications						
SRM 1588a (1989–1998)[b]	0	5	10	0	24	14
SRM 1649a (1982–1998)	5 (9)	0	0	22 (22)	35	8 (1)
SRM 1650a (1985–1999)	5 (6)	0	0	19 (25)	0	0
SRM 1939a (1990–1998)	(5)	3 (12)	0	0	20 (4)	3
Renewals						
SRM 1941a (1989–1994)[c]	11 (24)	0 (15)	0 (7)	23 (14)	21 (7)	6 (3)
SRM 1974a (1990–1995)	9 (19)	0 (13)	0 (12)	15 (18)	20 (4)	7 (4)

[a] The first number indicates the number of certified constituents; the number in parentheses indicates the number of noncertified or reference values.

[b] The first date indicates the year of the original certification and the second date is the year of the reissue of the material after recertification.

[c] The first date indicates the year of the original certification and the second date is the year of the issue of the renewal material.

TABLE 15.18

COMPARISON OF CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED PAHs IN SRM
1649a vs. SRM 1649

	SRM 1649a[a] (mg/kg) (%)	SRM 1649[b] [c] (mg/kg) (%)	Percent difference[d]	Overlap of uncertainty[e]
Phenanthrene	4.14 ± 0.37	(4.5 ± 0.3)	−8	X
Fluoranthene	6.45 ± 0.18 (2.7)[f]	7.1 ± 0.5 (7)[f]	−9	X
Pyrene	5.29 ± 0.25	(7.2 ± 0.2)[g]	−27	
		(6.3 ± 0.4)[h]	−16	
Benz[a]anthracene	2.21 ± 0.073 (3.3)	2.6 ± 0.3 (12)	−15	
Chrysene	3.049 ± 0.060	(3.5 ± 0.1)	−13	
Triphenylene	1.357 ± 0.054	(1.7 ± 0.1)	−20	
Benzo[b]fluoranthene	6.45 ± 0.64	(6.2 ± 0.3)	+4	X
Benzo[k]fluoranthene	1.913 ± 0.031	(2.0 ± 0.1)	−4	X
Benzo[e]pyrene	3.09 ± 0.19	(3.3 ± 0.2)	−6	X
Benzo[a]pyrene	2.509 ± 0.087 (3.5)	2.9 ± 0.5 (17)	−13	X
Perylene	0.646 ± 0.075	(0.84 ± 0.09)[g]	−19	
		(0.65 ± 0.02)[h]	0	X
Benzo[ghi]perylene	4.01 ± 0.91 (23)	4.5 ± 1.1 (24)	−11	X
Indeno[1,2,3-cd]pyrene	3.18 ± 0.72 (23)	3.3 ± 0.5 (15)	−4	X
Dibenz[a,h]anthracene	0.288 ± 0.023	0.41 ± 0.07	−30	

[a] Concentrations reported on dry mass basis; material as received contains approximately 1.2% moisture.

[b] Concentrations reported on an as received basis.

[c] Concentrations in parentheses were provided as noncertified (reference) values.

[d] Percent difference in the original certified values for SRM 1649 and the certified values for the reissue SRM 1649a.

[e] Overlap of uncertainties associated with the certified values or, in the case of the noncertified values for SRM 1649, overlap with the measurement uncertainty.

[f] Values in parentheses are the percent relative uncertainty of the certified values for the PAHs that were certified in the original SRM 1649.

[g] Result determined by GC–FID.

[h] Result determined by LC–FL.

PAHs and one nitro-PAH and noncertified (reference) concentrations for six PAHs, three nitro-PAHs, and a PAH ketone. This SRM was recently reanalyzed to provide certified and reference concentrations for 19 and 25 PAHs, respectively (see Tables 15.8 and 15.11). In the original certification analyses only GC/MS and LC–FL were used for the determination of the PAHs, whereas the recertification of SRM 1650a involved data sets from two to five different analytical techniques as described above for SRM 1649a. In Table 15.20 the results of the original certification are compared to the new certified values for PAHs. For all the original PAHs that had certified values, the uncertainties of the new certified values have decreased with the exception of benzo[a]pyrene where the uncertainties are similar. In contrast to the consistent decrease in the certified values for SRM 1649a shown in Table 15.18, for SRM 1650a the differences in concentrations for the original five PAHs with certified values are very small except for benzo[a]pyrene (+13%) and benzo[ghi]perylene (+170%). The large increase in concentration for benzo[ghi]perylene and indeno[1,2,3-cd]pyrene can be attributed to increased extraction efficiency in the recent analyses. For

TABLE 15.19

REFERENCE CONCENTRATIONS FOR SELECTED PCDD AND PCDF CONGENERS IN SRM 1649a URBAN DUST AND SRM 1944 NEW YORK/NEW JERSEY WATERWAY SEDIMENT

PCDD/PCDF Congeners	Concentration (μg/kg)	
	SRM 1649a	SRM 1944
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	0.011 ± 0.004	0.133 ± 0.009
1,2,3,7,8-Pentachlorodibenzo-*p*-dioxin	0.091 ± 0.012	0.019 ± 0.002
1,2,3,4,7,8-Hexachlorodibenzo-*p*-dioxin	0.26 ± 0.02	0.026 ± 0.003
1,2,3,6,7,8-Hexachlorodibenzo-*p*-dioxin	0.68 ± 0.05	0.056 ± 0.006
1,2,3,7,8,9-Hexachlorodibenzo-*p*-dioxin	0.64 ± 0.11	0.053 ± 0.007
1,2,3,4,6,7,8-Heptachlorodibenzo-*p*-dioxin	18.8 ± 1.5	0.80 ± 0.07
Octachlorodibenzo-*p*-dioxin	201 ± 20	5.8 ± 0.7
2,3,7,8-Tetrachlorodibenzofuran	0.068 ± 0.015	0.039 ± 0.015
1,2,3,7,8-Pentachlorodibenzofuran	0.090 ± 0.010	0.045 ± 0.007
2,3,4,7,8-Pentachlorodibenzofuran	0.28 ± 0.03	0.045 ± 0.004
1,2,3,4,7,8-Hexachlorodibenzofuran	0.87 ± 0.26	0.22 ± 0.03
1,2,3,6,7,8-Hexachlorodibenzofuran	0.43 ± 0.06	0.09 ± 0.01
2,3,4,6,7,8-Hexachlorodibenzofuran	0.69 ± 0.03	0.054 ± 0.006
1,2,3,7,8,9-Hexachlorodibenzofuran	0.066 ± 0.029	0.019 ± 0.018
1,2,3,4,6,7,8-Heptachlorodibenzofuran	3.8 ± 0.2	1.0 ± 0.1
1,2,3,4,7,8,9-Heptachlorodibenzofuran	0.46 ± 0.07	0.040 ± 0.006
Octachlorodibenzofuran	6.8 ± 0.8	1.0 ± 0.1
Total toxic equivalents (TEQ)[a]	1.00 ± 0.07	0.25 ± 0.01
Total tetrachlorodibenzo-*p*-dioxins	0.16 ± 0.08	0.25 ± 0.05
Total pentachlorodibenzo-*p*-dioxins	0.88 ± 0.16	0.19 ± 0.06
Total hexachlorodibenzo-*p*-dioxins	6.0 ± 0.5	0.63 ± 0.09
Total heptachlorodibenzo-*p*-dioxins	36.4 ± 3.4	1.8 ± 0.2
Total tetrachlorodibenzofurans	0.52 ± 0.13	0.7 ± 0.2
Total pentachlorodibenzofurans	1.6 ± 0.2	0.74 ± 0.07
Total hexachlorodibenzofurans	5.0 ± 0.6	1.0 ± 0.1
Total heptachlorodibenzofurans	9.8 ± 0.8	1.5 ± 0.1
Total dibenzo-*p*-dioxins[b]	244 ± 22	8.7 ± 0.9
Total dibenzofurans[b]	23.9 ± 1.9	5.0 ± 0.5

[a] TEQ is the sum of the products of each of the 2,3,7,8-substituted congeners multiplied by their individual toxic equivalency factors (TEFs) recommended by the North Atlantic Treaty Organization (NATO) [56].

[b] Total of tetra- through octa-substituted PCDD and PCDF congeners.

the original certification measurements, the samples were Soxhlet extracted with toluene/methanol and dichloromethane. In a recent study by Schantz et al. [22] comparing Soxhlet extraction and pressurized fluid extraction (PFE) for the extraction of natural matrix SRMs, they found that for the diesel particulate materials (SRMs 1650 and 2975) the PFE extracted 20–50% more of the higher molecular weight PAHs such as benzo[*ghi*]-perylene and indeno[1,2,3-*cd*]pyrene than Soxhlet extraction. Recent analyses of SRM 1650 using Soxhlet extraction and GC/MS and LC–FL have also found higher concentrations of benzo[*ghi*]perylene and indeno[1,2,3-*cd*]pyrene compared with the original certification measurements, indicating that the original measurements for these compounds

TABLE 15.20

COMPARISON OF CERTIFIED AND REFERENCE CONCENTRATIONS FOR SELECTED PAHs IN SRM 1650a vs. SRM 1650

	SRM 1650a (mg/kg) (%)	SRM 1650[a] (mg/kg) (%)	Percent difference[b]	Overlap of uncertainty[c]
Phenanthrene	68.4 ± 8.5	(79 ± 1)	−16	
		(63 ± 2)	+7.8	X
Fluoranthene	49.9 ± 2.7 (5.4)[d]	51 ± 4 (7.8)[d]	−2.2	X
Pyrene	47.5 ± 2.7 (5.8)	(48 ± 4) (8.3)	−1	X
Benz[a]anthracene	6.33 ± 0.77 (12)	6.5 ± 1.1 (17)	−2.7	X
Chrysene	14.45 ± 0.84	(22 ± 1)	−34	
Benzo[k]fluoranthene	2.64 ± 0.31	(2.1 ± 0.2)	+26	X
Benzo[e]pyrene	7.44 ± 0.53	(9.6 ± 0.3)	−22	
Benzo[a]pyrene	1.33 ± 0.35 (27)	1.2 ± 0.3 (25)	−13	X
Perylene	0.157 ± 0.044	(0.13 ± 0.02)	+21	
Benzo[ghi]perylene	6.50 ± 0.94 (15)	2.4 ± 0.6 (25)	+170	
Indeno[1,2,3-cd]pyrene	5.62 ± 0.53	(3.2 ± 0.5)[e]	+75	
		(1.8 ± 0.1)[f]	+173	
		(2.1 ± 0.1)[g]	+167	

[a] Concentrations in parentheses were provided as noncertified (reference) values.

[b] Percent difference in the original certified values for SRM 1650 and the certified values for the reissue SRM 1650a.

[c] Overlap of uncertainties associated with the certified values or, in the case of the noncertified values for SRM 1650, overlap with the measurement uncertainties.

[d] Values in parentheses are the percent relative uncertainty of the certified values for the PAHs that were certified in the original SRM 1650.

[e] Result determined by LC–FL.

[f] Result determined by GC/MS (electron impact).

[g] Result determined by GC/MS (negative ion chemical ionization).

were probably low due to incomplete extraction from the particulate matter. For the assignment of the new certified values for benzo[ghi]perylene, indeno[1,2,3-cd]pyrene, and several other high molecular weight PAHs, only results obtained from PFE were used.

15.5.1.3 Recertification of SRM 1588a Organics In Cod Liver Oil

As was the case with the PAHs, the analytical approach for the certification of PCB congeners and chlorinated pesticides has also improved since the first generation of natural matrix SRMs to increase the number of analytes determined. Two SRMs certified in the late 1980s only for PCB congeners and pesticides, SRM 1588 Organics in Cod Liver Oil and SRM 1939 PCB Congeners in River Sediment, were recertified and updated 8–9 years after the original certification. In the original certifications for both of these SRMs, only a limited number of PCB congeners and pesticides were certified (see Table 15.17) using GC–ECD and GC/MS. Using the current analytical approach, SRM 1588a and SRM 1939a have certified values for 39 and 23 PCB congeners and pesticides, respectively.

Table 15.21 compares the certified values for the compounds in the original certification

TABLE 15.21

COMPARISON OF CERTIFIED CONCENTRATIONS FOR PCB CONGENERS AND CHLORINATED PESTICIDES IN SRM 1588a vs. 1588

	SRM 1588a (µg/kg wet-basis)	Percent relative uncertainty	SRM 1588 (µg/kg wet-basis)	Percent relative uncertainty	Percent difference[a]
PCB 101/90	126.5 ± 4.3	(3.4)	129 ± 5	(3.9)	−1.9
PCB 138/163/164	263.5 ± 9.1	(3.5)	261 ± 29	(11)	+1.0
PCB 153	273.8 ± 7.7	(2.8)	276 ± 40	(14)	−0.8
PCB 170/190	46.5 ± 1.1	(2.4)	45 ± 5	(11)	+3.3
PCB 180	105.0 ± 5.2	(5.0)	107 ± 4	(3.7)	−1.9
Hexachlorobenzene	157.8 ± 5.0	(3.2)	148 ± 21	(14)	+6.6
α-HCH	85.3 ± 3.4	(4.0)	86 ± 19	(22)	−0.8
cis-Chlordane	167.0 ± 5.0	(3.0)	158 ± 8	(5.0)	+5.7
trans-Nonachlor	214.6 ± 7.9	(3.7)	209 ± 11	(5.3)	+2.7
Dieldrin	155.9 ± 4.5	(2.9)	150 ± 12	(8.0)	+3.9
4,4'-DDE	651 ± 11	(1.7)	641 ± 62	(9.7)	+1.6
4,4'-DDD	254 ± 11	(4.3)	277 ± 15	(5.4)	−8.3
2,4'-DDT	156.0 ± 4.4	(2.8)	156 ± 5	(3.2)	0.0
4,4'-DDT	524 ± 12	(2.3)	529 ± 45	(8.5)	−1.0

[a] Percent difference in the original certified values for SRM 1588 and the certified values for the reissue SRM 1588a.

of SRM 1588 with the certified values from the recertification (i.e., SRM 1588a). No significant or consistent differences were observed between the old and the new certified values. Only three compounds had certified values differing by more than 5%, with the greatest difference at −8.3% for 4,4'-DDD, indicating that the material has been stable since the original measurements. However, for all of the certified values in SRM 1588a, the uncertainties (typically 3–5%) were decreased compared with the uncertainties for the certified values for SRM 1588 (typically 5–15%). In addition to the certified values, reference values, which are based on measurements performed outside NIST, are reported for 37 additional PCB congeners and pesticides.

15.5.2 Renewal SRMs

When preparing a natural matrix SRM, NIST will typically attempt to prepare sufficient material for a projected 7–8 year supply. When the material is depleted a new batch of material is prepared, analyzed, value assigned, and issued as a renewal material. In general the goals for a renewal of a natural matrix SRM are similar to those described above for a recertification, i.e., (1) to increase the number of certified and reference concentrations for PAHs, PCBs, pesticides, and other analytes, (2) to reduce the uncertainties associated with the certified concentrations, and (3) to provide sufficient material to last for 7–8 years.

15.5.2.1 Marine sediment and mussel tissue SRMs

Two of the most popular natural matrix materials, SRM 1941 Organics in Marine

TABLE 15.22

COMPARISON OF CERTIFIED CONCENTRATIONS FOR SELECTED PAHs IN SEDIMENT AND MUSSEL TISSUE: ORIGINAL CERTIFICATION vs. RENEWAL CERTIFICATION

	SRM 1941 (μg/kg) (%)	SRM 1941a (μg/kg) (%)	SRM 1974 (μg/kg wet-basis) (%)	SRM 1974a (μg/kg wet-basis) (%)
Phenanthrene	577 ± 59 (10)[a]	489 ± 23 (5)	45 ± 11 (24)	22.2 ± 2.4 (11)
Anthracene	202 ± 42 (20)	184 ± 14 (8)	6.1 ± 1.7 (28)	6.1 ± 1.7 (28)
Fluoranthene	1220 ± 240 (20)	981 ± 78 (8)	272 ± 47 (17)	163.7 ± 9.1 (5.6)
Pyrene	1080 ± 200 (18)	811 ± 24 (3)	276 ± 30 (11)	151.6 ± 6.6 (4.3)
Benz[a]anthracene	550 ± 79 (14)	427 ± 25 (6)		32.5 ± 4.7 (14)
Benzo[b]fluoranthene	780 ± 190 (24)	740 ± 110 (15)	52.3 ± 9.4 (18)	46.4 ± 3.7 (8)
Benzo[k]fluoranthene	444 ± 49 (11)	361 ± 18 (5)		20.18 ± 0.84 (4.2)
Benzo[a]pyrene	670 ± 130 (19)	628 ± 51 (8)	18.6 ± 3.8 (20)	15.63 ± 0.65 (4.2)
Perylene	422 ± 33 (8)	452 ± 58 (13)	8.5 ± 2.4 (28)	7.68 ± 0.27 (3.5)
Benzo[ghi]perylene	516 ± 83 (16)	525 ± 67 (13)	20.0 ± 2.3 (12)	22.0 ± 2.2 (10)
Indeno[1,2,3-cd]pyrene	569 ± 40 (7)	501 ± 72 (14)	14.6 ± 2.7 (18)	14.2 ± 2.8 (20)

[a] Values in parentheses are the percent relative uncertainty of the certified values.

Sediment and SRM 1974 Organics in Mussel Tissue, were renewed after supplies were exhausted in 5 years (see Table 15.17). The renewals for both of these materials represent the second generation of SRMs as far as the analytical approach for value assignment of PAHs, PCBs, and pesticides. As shown in Table 15.17, the number of compounds with certified values increased significantly for SRMs 1941a and 1974a compared to the original materials. In fact SRM 1941 and SRM 1974 were originally issued in 1989 and 1990, respectively, with no certified values for PCB congeners and pesticides, i.e., only noncertified (reference) values were provided based on results from one analytical method. In Table 15.22 the certified values and the uncertainties for 11 PAHs (those originally certified in SRM 1941) are compared for the original certification versus the renewal certification for both SRM 1941 and SRM 1974. The comparison illustrates the similar concentrations in the original and renewal materials, which were both collected at the same sites, i.e., the sediment in Baltimore (Maryland) harbor and the mussels in Boston (Massachusetts) harbor. As discussed above for the recertification of the existing SRMs, the analytical methods and approach improved sufficiently between the original versus renewal certification measurements to reduce the uncertainties associated with the certified values in the renewal materials for all the PAHs except for indeno[1,2,3-cd]pyrene in both the sediment and mussel tissue. The percent relative uncertainties for the original materials ranged from 7% to 24% for the sediment and from 11% to 28% for the mussel tissue, whereas for the renewal materials the uncertainties ranged from 3% to 14% (with 7 of the 11 less than 10%) for SRM 1941a, and from 4% to 20% (with only 4 greater than 10%) for the SRM 1974a. Both the increase in the number of PAHs certified and the decrease in the uncertainties associated with the certified values for the renewal materials illustrate the improvements in the second generation of natural matrix SRMs.

Because of its popularity, the supply of SRM 1941a will be depleted by the end of 1999. The replacement material, SRM 1941b, has been collected from the same Baltimore

harbor site, and certification measurements are now in progress. We plan to collect the replacement material for SRM 1974a within the next year.

15.5.2.2 SRM 1589a PCBs in Human Serum

SRM 1589 Polychlorinated Biphenyls (as Aroclor 1260) in Human Serum was issued in 1985 with a certified concentration of Aroclor 1260 (i.e., total PCBs). SRM 1589 was prepared by the addition of a known quantity of Aroclor 1260 to a human serum pool. Since SRM 1589 was issued, the scientific community has focused on the measurement of individual PCB congeners rather than total PCBs as an Aroclor mixture. Measurements of selected congeners are of more interest as indicators of PCB contamination (e.g., PCB 138, 153, 170, and 180) or because of their relative toxicity (e.g., the planar congeners, PCB 77, 126, and 169). Most researchers using SRM 1589 indicated that the renewal material should not be prepared by the addition of Aroclor to serum because this does not reflect the metabolized pattern for the PCB congeners. To meet the changing needs of the users, the renewal material, SRM 1589a, was prepared by obtaining human serum with relatively high natural levels of PCB contamination. Measurements are in progress to determine individual PCB congeners using GC–ECD and GC/MS. Results from the Centers for Disease Control and Prevention (Atlanta, GA) for PCBs, chlorinated pesticides, and PCDD and PCDF congeners will be used in conjunction with NIST measurements to determine the certified and reference values for the natural levels of these analytes in the SRM. Even though this is a renewal SRM, the preparation and measurement approach for SRM 1589a are sufficiently different to consider this as a new material in many respects.

15.5.3 New SRMs

Since the previous review of environmental SRM activities [17], several new materials have been developed including whale blubber, a contaminated marine sediment, diesel particulate matter and diesel particulate extract, and three freeze-dried mussel tissue materials (see Table 15.4). Each of these materials is described in more detail below.

15.5.3.1 SRM 1945 Organics in Whale Blubber

SRM 1945 Organics in Whale Blubber, which was issued in 1994, was the first natural matrix SRM certified for PCB congeners and pesticides using the current analytical approach described above, and it is the SRM with the greatest number of certified values for PCB congeners and chlorinated pesticides (i.e., 42) [14]. This material was developed at the request of the National Oceanic and Atmospheric Administration to provide quality assurance in the measurement of organic contaminants in marine mammal tissues. This material represents a high lipid matrix (~74% extractable mass) and is provided as a frozen tissue homogenate similar to SRM 1974a.

15.5.3.2 SRM 1944 New York/New Jersey Waterway Sediment

SRM 1944 New York/New Jersey Waterway Sediment, a highly contaminated marine

sediment, was issued in 1999 in paɪ́ɩ to meet the needs of laboratories involved in the testing of sediment dredged ɪɾom waterways and harbors to measure contaminant levels to determine the appropriate disposal methods. The levels of PAHs, PCBs, and pesticides are 5–10 times greater in SRM 1944 compared to SRM 1941a (see Tables 15.5, 15.12 and 15.15). SRM 1944 is also one of the first natural matrix SRMs that has certified concentrations for both organic contaminants and trace elements. Several of the previous organic contaminant SRMs had reference values for trace elements (e.g., SRMs 1649, 1941 and 1974 and the renewal materials SRMs 1649a, 1941a, and 1974a). SRM 1944 is also one of the first NIST SRMs (with SRM 1649a) to have values assigned for the concentrations of the seventeen 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners and total tetra-, penta-, hexa-, and hepta-congeners of polychlorinated dibenzo-*p*-dioxin and dibenzofuran. Reference values were determined from an interlaboratory comparison exercise among 14 laboratories and the results are summarized in Table 15.19.

15.5.3.3 New diesel particulate matter SRMs

Two new diesel particulate-related SRMs have been developed to complement the existing diesel particulate material, SRM 1650a (see above). Bulk quantities of a new diesel particulate material were obtained from the filtering system of an industrial forklift. This material had been identified by EPA as a diesel particulate material with relatively high mutagenic activity. SRM 2975 Diesel Particulate Matter (Industrial Forklift) was prepared from this bulk material, and a portion of this same bulk diesel particulate material was extracted with dichloromethane to prepare SRM 1975 Diesel Particulate Extract. SRM 1975 was developed in response to the needs of both the environmental analytical and bioassay communities for a diesel particulate extract SRM to eliminate the variability associated with the solvent extraction of the particulate material. In addition to certified and reference concentrations for PAHs (see Tables 15.7 and 15.10) and nitro-PAHs, SRM 1975 also has reference values for mutagenic activity determined by EPA [42].

15.5.3.4 Mussel tissue materials

Three freeze-dried mussel materials have been developed to complement SRM 1974a, which is provided as a frozen tissue homogenate, and to provide materials with different levels of contaminants. SRM 2974 Organics in Freeze-Dried Mussel Tissue was prepared from the same batch of mussel tissue homogenate as SRM 1974a. Thus, with the exception of several of the more volatile components, of which some may have been lost during the freeze-drying process, the certified values for SRM 2974 are identical to the values for SRM 1974a on a dry-mass basis. The other two freeze-dried mussel tissue materials, SRM 2977 Mussel Tissue (Organic Contaminants and Trace Elements) and SRM 2978 Mussel Tissue (Organic Contaminants - Raritan Bay, NJ) were developed as a joint international effort in response to the recommendations of the Group of Experts on Standards and Reference Materials (GESREM) established by the Intergovernmental Oceanographic Commission (IOC), United Nations Environment Program (UNEP), and the International Atomic Energy Agency (IAEA). SRM 2978 was prepared from mussels collected in

Raritan Bay, New Jersey and has contaminant levels similar to SRM 2974. SRM 2977, which was prepared from mussels collected in Guanabara Bay, Brazil, has contaminant levels 3–10 times lower than SRM 2974.

15.5.4 New SRMs in progress

A fish tissue material, SRM 1946 Lake Superior Fish Tissue, which will be issued as a frozen tissue homogenate prepared similar to SRM 1974a and SRM 1945, is currently in progress. Approximately 80 kg of filets from lake trout from Lake Superior were cryogenically pulverized and homogenized to prepare SRM 1946. This SRM will be certified for PCBs, pesticides, methylmercury, and total mercury, with the possible addition of reference values in the future for PCDDs, PCDFs, and toxaphene. Because the fish tissue SRM can also be considered as a food matrix, we will also provide measurements on food proximates (solids, ash, fat, nitrogen, protein, and carbohydrates), fatty acids, and calories to make the SRM useful for the food measurement community.

15.5.5 New analytes in existing SRMs

To date most of the NIST activities for natural matrix SRMs for organic contaminants have focused on measurements for selected PAHs, PCB congeners, and chlorinated pesticides. The need for SRMs with certified and reference values for other classes of contaminants is increasing. PCDDs and PCDFs are one of the most toxic group of organic contaminants; however until the mid-1990s there were no environmental matrix CRMs with natural levels (i.e., not fortified) for PCDDs and PCDFs from any reference material-producing organization. Since 1995 four CRMs have been issued by three different organizations including a fish tissue (National Research Council of Canada, CARP-1) [43], two Great Lakes sediments (Environment Canada, National Water Research Institute)[44], and milk powder (CRM 607) [45] and fly ash (CRM 490) [46] from the Standards, Measurements and Testing (SMT) Programme of the European Commission. Because of the lack of natural matrix CRMs with concentration values assigned for PCDDs and PCDFs prior to 1995, Nestrick et al. [47,48] proposed in 1983 the use of two existing NIST air particulate matter SRMs, SRMs 1648 and 1649, as reference materials for these measurements. SRM 1648 Urban Particulate Matter, which has certified concentrations for selected inorganic constituents, was collected in the mid-1970s in St. Louis, MO. SRM 1649, Urban Dust/Organics, which as mentioned above was originally issued in 1982 with certified concentrations for several PAHs, was collected in 1976–77 in Washington, DC. Nestrick et al. [47,48] suggested that these two materials could serve as reference materials because as existing SRMs they had been determined to be homogeneous for other constituents, and they were available at a reasonable cost. They reported values for 22 tetrachloro-, 10 hexachloro-, 2 heptachloro, and octachloro-substituted dibenzo-*p*-dioxin congeners, and 2,3,7,8-tetrachlorodibenzofuran in both of these SRMs. Because of the limited availability of CRMs for PCDD and PCDF measurements, an interlaboratory study was undertaken by NIST and the Analysis and Air Quality Division, Environmental Technology Centre, Environment Canada to establish some reference concentration values for selected PCDD and PCDF congeners in two

existing SRMs, SRM 1649a and SRM 1944. Results from the interlaboratory study were used to provide reference values for the seventeen 2,3,7,8-substituted polychlorinated dibenzo-*p*-dioxin and dibenzofuran congeners and total tetra-, penta-, hexa-, and hepta-congeners of polychlorinated dibenzo-*p*-dioxin and dibenzofuran in both of these SRMs (see Table 15.19).

Another class of chlorinated hydrocarbon analytes of interest are the PCB congeners without *ortho*-substituted chlorines (known as non-*ortho* or 'coplanar' PCBs) because they are potentially the most toxic PCBs [49]. Over the past decade LC methods have been developed to isolate the non-*ortho* PCBs from other interfering PCBs [50,51] which then allows for subsequent analysis by GC–ECD or GC/MS. Measurements of non-*ortho* PCBs have now been reported in a variety of environmental matrices, including sediments, fish, and marine mammals. However, at present there are no SRMs or CRMs from other sources with certified concentrations for these important PCB congeners. To meet this need, NIST is currently analyzing four existing marine matrix SRMs (SRM 1588a, SRM 1944, SRM 1945, and SRM 1974a) to assign certified and/or reference values for PCB 77, PCB 126, and PCB 169.

As described above, PAHs have been one of the primary groups of compounds measured in environmental matrix SRMs. However, their sulfur analogues, polycyclic aromatic sulfur heterocycles (PASHs), have been neglected even though their occurrence in various fossil fuels and other environmental samples as well as their mutagenic and carcinogenic potential have been reported [52]. Recently Mössner and Wise [53] reported the determination of selected PASHs in SRM 1597 Complex Mixture of PAHs from Coal Tar and SRM 1582 Petroleum Crude. They reported the concentrations in these two SRMs for dibenzothiophene, naphtho[1,2-*b*]thiophene, naphtho[2,1-*b*]thiophene, naphtho[2,3-*b*]thiophene, benzo[*b*]naphtho[1,2-*d*]thiophene, benzo[*b*]naphtho[2,1-*d*]thiophene, and benzo[*b*]naphtho[2,3-*d*]thiophene, as well as concentrations for a number of the methyl-substituted isomers of the above-mentioned PASHs.

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Chapter 16

Interpretation of environmental data using chemometrics

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CONTENTS

16.1 INTRODUCTION

Assessment of environmental data plays an important role in any sustainable develop-
ment policy. Chemometrics provides powerful tools for the analysis of the large environ-
mental multivariate data sets obtained by modern analytical instrumentation and
laboratories. The environmental analyst is increasingly faced with the need of using
more sophisticated mathematical and statistical methods in his daily work. The new
information technologies and data processing capabilities of personal computers present

in nearly all analytical and environmental laboratories worldwide increase the potentialities of chemometric applications. Apart from general chemometric textbooks [1–5], some monographs dedicated to chemometric analysis of environmental chemistry and environmental analysis data have recently appeared [6–8]. Software for chemometrics is also available [9–11].

The interpretation of complex multivariate environmental data is challenging for several reasons [12]. On the one hand it requires accurate chemical analysis, frequently at trace or subtrace levels. On the other hand, the deduction of spatial and temporal distribution of chemicals in environment is hampered by the usually limited amount of available data, and by the limited knowledge about their sources. Finally, the data collected experimentally are usually non-selective. Multivariate statistical data analysis and chemometrics methods can be used to solve these problems. In this chapter, some of the more frequently used methods in the chemometrics literature for the analysis of environmental data are briefly described and some references are given for further study. Recent applications of these chemometric methods to environmental data are included and a new exploratory approach for environmental data analysis, multivariate curve resolution (MCR), is presented and applied to several problems related to mixture resolution and pollution source input identification and apportionment.

16.2 MULTIVARIATE ANALYSIS OF ENVIRONMENTAL DATA

In multivariate analysis of environmental data, different aspects are considered, depending on the type of data and on the goals of the analysis. In this chapter, three aspects are considered:

(a) aspects related to environmental data structures;
(b) aspects related to multivariate exploratory data analysis and pattern recognition techniques; and
(c) aspects related to data modeling, such as classification and calibration.

Additionally, a new approach for mixture resolution of environmental sources of data variation is presented and described in more detail. In Section 3, some application examples of this approach to environmentally related problems are given.

16.2.1 Environmental data

Environmental data is characterized by inherent variability. Only limited understanding of the environmental distribution of contaminants can therefore be recovered from a single analysis. The variability of environmental data can be divided into three main sources:

(a) natural sources without strong contamination influences, changing geographically and temporally in a stochastic way as a consequence of natural phenomena;
(b) anthropogenic sources interfering with natural variability and caused by anthropogenic activities;
(c) experimental error sources caused by the different steps of the analytical process: sampling, sample pretreatment, sample measurement and data evaluation.

The contribution of experimental error to data variability increases when the sample size decreases.

Usually it is difficult to have measurements totally selective for the components to be analyzed or for their sources. Apart from background noise, experimental data are affected by chemical and/or physical interferences from the samples or from the measurement process. In the measuring process non-linear phenomena can cause additional problems. Traditionally chemical interferences are excluded to assure the selectivity of the measurement process and a linear response is searched in a short interval range of instrument scales. In some cases this is an expensive task because of time or economic reasons. In other cases the interest is in the quantitation of several components of the same sample, whether interferents or not, and therefore their previous elimination is not of interest. In all these cases, the use of univariate data analysis techniques is totally inappropriate and more multivariate data analysis techniques are required.

Univariate data are obtained from analytical signals measured as a function of a unique control variable, i.e. wavelength, time, etc. Obviously, the amount of information that can be extracted from univariate signals is limited. For instance, in liquid chromatography with UV detection at a single wavelength, the deduction of the number of coeluted compounds in a complex chromatographic peak is not possible. These difficulties can be overcome by measuring multivariate signals instead, as for instance in the case of liquid chromatography by the introduction of multivariate diode array UV spectrometric detectors, where measurements are performed simultaneously at several wavelengths (the obtained signal is multivariate instead of univariate). Moreover, the objectives in environmental analysis are usually multivariate. Several contamination patterns from different, often unknown, sources may occur. The state of pollution of a particular sampling site depends mostly on the nature of multiple different sources of pollution; e.g. wastewater effluents contain different contaminants, ranging from heavy metals to organic compounds. If the presence and effects of different contaminants, their potential reactions and interactions are to be simultaneously studied and interpreted, the application of chemometric multivariate methods is required. Several advantages are obtained from multivariate experimental data if the proper multivariate data analysis tools are applied. In the present chapter some of these tools are described and examples of their application are shown.

According to the complexity of the data structures, other more precise data classifications have been proposed, taken from tensor theory and multiway data analysis methods. Booksh and Kowalski [13] give a theoretical description of multivariate data structures in analytical chemistry and refs. [6–8] give examples of multivariate environmental analytical data. In Fig. 16.1, an example with a graphical representation of a data matrix (two-way data) obtained during the coelution of four analytes measured by LC–DAD is given. The columns of the data matrix are the spectra measured at different elution times and the rows of this matrix are the chromatograms measured at each wavelength. Both rows and columns are correlated, i.e., during the elution of the four coeluting components, their concentration will change in accordance with the elution process and very probably their UV pure spectra are also very similar. If all the columns of the data matrix are simultaneously considered, more information can be extracted than if only one column is considered. This is the task of the multivariate techniques: the simultaneous manipulation of
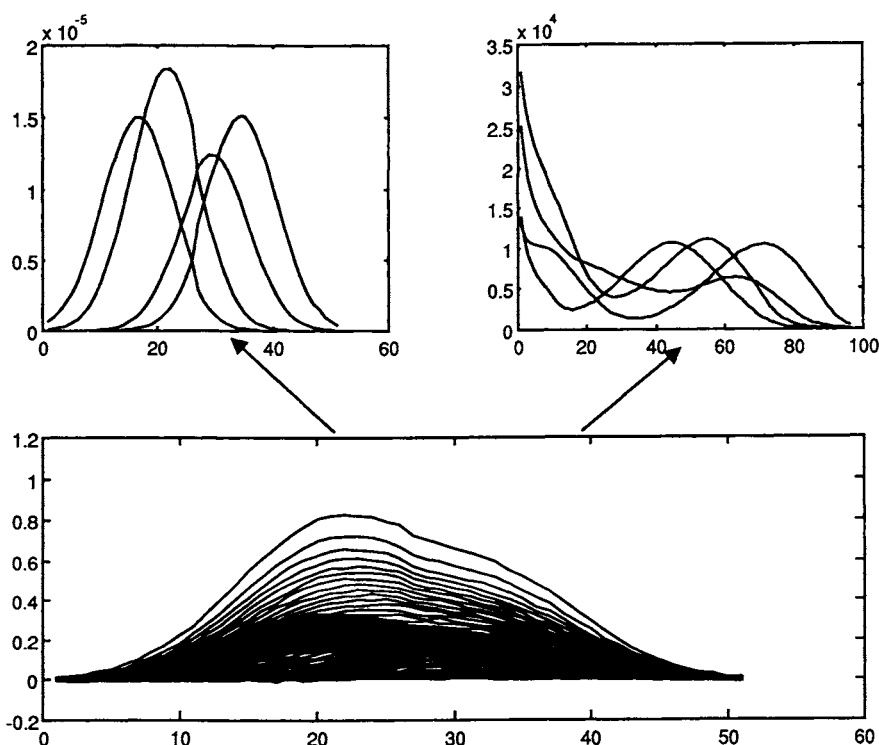
Fig. 16.1. The coelution of four components in LC–DAD gives a two-way data matrix, whose columns are the chromatograms at each wavelength (down) and whose rows are the DAD spectra at each elution time. The columns of the data matrix are a linear combination of the elution profiles of the four components (left up) and the rows of the data matrix are a linear combination of the pure spectra of the four components (right up).

many variables (the whole data matrix) to extract maximum information. From a mathematical point of view, the data table is a data matrix of numbers, and the statistical techniques needed to process these multivariate data require linear algebra formulations. In this particular case, the data table is represented by D, which refers to a data matrix with NR rows and NC columns. If several data tables or data matrices with correlated information are analyzed simultaneously, a new data structure or higher order tensor (cube or hypercube) must be considered (Fig. 16.2). As most of the mathematical operations with these data sets require the use of linear algebra, a convenient way to transform these higher order or multiway data sets to data matrices is by means of data unfolding (Fig. 16.2).

Analytical signals are made up of relevant parts, background and noise. The relevant parts of the signal are frequently peak shaped and have an intermediate frequency. Electronic noise (from analytical instruments) often has a much higher frequency than the relevant parts of the signal. At lower frequencies than the relevant parts of the signal other undesired features of the analytical process appear such as trends, drifts or shifts of the baseline. Many signal and data pretreatments try to capitalize on the differences in the frequency domain between the useful and the undesirable parts of the raw signal. Data
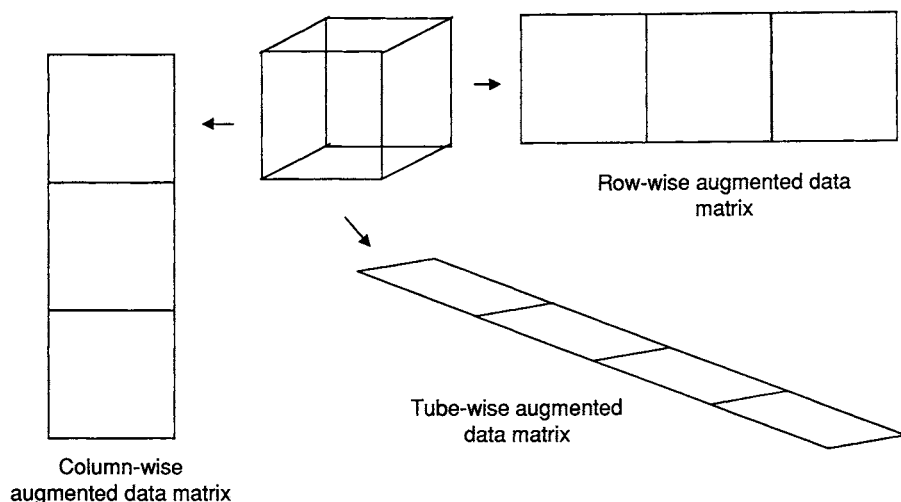
Fig. 16.2. A three-way data set or data cube may be unfolded in three different ways, giving respectively an augmented row-wise data matrix, an augmented column-wise data matrix or an augmented tube-wise data matrix.

transformation and preprocessing techniques needed to extract environmental information from experimental data, depend on the way the data have been obtained, on the type of information sought and on the previous knowledge of the analyzed system. There is not a single method able to process every data type. In many cases, data transformation allows a better extraction of the information sought. Examples of data transformation techniques are derivatives, digital noise filtering and smoothing, Fourier transform, log transformation, normalization and subtraction. Derivatives and smoothing are usually based on Savitzky-Golay polynomial filtering [14,15]. This method applies a convolution filter to every data point by considering *n* points on either side of the center point, where a polynomial is fitted. First-derivative data treatment eliminates constant contributions. Second-derivative treatment removes also first order (slope lines) constant contributions. Derivative transformations usually enhance data resolution and are useful to identify overlapped signals. However, derivative transformations also produce noise enhancement, although this can be partly overcome by noise filtering and smoothing. It is not common to work with higher order derivatives because of the overly high noise contribution to the signal. Fourier transform techniques are used in some spectrometric methods and also for noise filtering and deconvolution [16,17]. Log transformation is often used to emphasize low intensity values or to change data distributions [18]. A very well known log10 transformation is the conversion of transmittance spectra, which are not linear with concentration, into absorbance spectra, which are linear with concentration.

Normalization is a very common data transformation and there are many types. One of the more typical normalizations is the equal vector length normalization, where each value in a sample is divided by the root square of the sum of the squares of all the variables measured for that sample. Normalization is used to compare samples where the amount of sample to analyze can vary. Subtraction, like derivatives, is another transformation

method, which can be used to remove background and bias. A constant amount can be subtracted to eliminate constant shifts or baselines, or a variable amount can be subtracted for each sample to eliminate slope baseline tendencies or trending. In that case, polynomia fitted to baseline points can be subtracted. A common subtraction procedure in some spectroscopies (like NIR) is data detrending. This transformation technique has similar effects to second derivative transformations, i.e. it subtracts baseline effects, but it does it differently to derivatives, since it applies just a polynomial subtraction and avoids noise enhancement and formation of new bands associated with derivatives. The signal is kept as it is and only the linear tendency is eliminated.

While the previously described data transformation techniques are applied on a sample basis (per sample), data preprocessing techniques are usually applied on a variable basis. The more common data preprocessing techniques are mean centering and variance scaling. Mean centering does a data translation, changing the data origin from zero to the mean data point. This allows a clear distinction of the differences from the average but on the other hand will obscure the apportionment of the sample content. Variance scaling is especially useful when data from different variables are expressed in different units and ranges. In that case obviously, the variable with the largest values will dominate the variance. Thus the variables with low values are masked. In many situations, it is desirable to scale the data to remove the effect of the dominating variables. It is not recommended, however, do data scaling for spectroscopic measurements, where the data from different variables are in the same units and highly correlated. Variance scaling is performed, dividing each data point by the standard deviation over each variable. In autoscaling, the data are mean-centered and then variance scaled. With this method, the data points became more equivalently distributed, centered at the mean origin. Other preprocessing techniques include range scaling, where all data points are scaled between zero and one, by subtraction of the minimum value and dividing by the maximum difference between data points. This preprocessing technique is usually performed for plotting purposes. Two additional problems frequently encountered in environmental data tables are the presence of a large number of missing values and of values below the limit of detection. Values below the limit of detection are usually assumed to be positive and equal to the LOD, or a fraction of it. Missing values can be substituted by some estimation of them using an appropriate method, as for instance principal component analysis (see below).

As a preliminary step of any study of environmental data tables, the correlation between variables is first investigated to see the interrelationships between the variation of the different analyte concentrations in the samples analyzed. This is accomplished simply by calculating the correlation coefficients between all the variables in the data sets available from raw data.

16.2.2 Exploratory data analysis

The goal of exploratory data analysis techniques is the computation and graphical display of patterns of association in multivariate data sets, looking for possible groupings and sources of data variation. Exploratory data methods allow a fast deduction of correlated variables, sample grouping and outlier detection. They also allow a powerful data screening of the data structure, which can be used in subsequent multivariate analysis steps. The algorithms for this exploratory task are designed to reduce large and complex

data sets into a set of best views of the data; these views provide insight into the data structure and data correlations that exist among the samples and variables. Factor analysis (FA) and especially, principal component analysis (PCA), play a fundamental role in this context [3].

16.2.2.1 Principal component analysis (PCA)

Principal component analysis allows the transformation of complex data sets into a new perspective in which, hopefully, the most important or relevant information is made more obvious. PCA has been used as an explorative tool to investigate the intrinsic dimensionality of a data set. In experimental data, there may be a reduced number of factors which influence the observed data variance, and these factors are not directly available from experimental measurements (hidden factors) because there are no specific sensors for them. To compensate for the lack of specific sensors, more data are collected from non-specific variables, which hopefully will reveal the hidden property of interest, by proper mathematical data treatment. A new set of orthogonal variables or principal components is obtained by linear combination of the original variables in the data set. These new variables are also called factors and allow the calculation of a new set of plotting axes, which are orthogonal and account for maximum explained data variance in decreasing order: the first factor explains more variance than the second factor, and so forth. The goal of PCA is to find this new set of orthogonal coordinate axes onto which we will project the data. This is in contrast to the original data where, in general, the variables are highly correlated. The procedure of matrix decomposition gives two matrices, the scores (U) matrix and the loadings (V) matrix:

$$\mathbf{D} = \mathbf{U}\mathbf{V}^{\mathrm{T}} \tag{1}$$

The product of scores and loadings will reproduce the original data $\mathbf{D}$. Loadings will give the contribution of the original variables to each factor direction. The coefficients of the loadings, the scores, are the transformation of the original responses in $\mathbf{D}$ into the new data space axes represented by the factor axes. Because the relevant information in a data set is concentrated in the first factors generated during PCA, it is often the case that a portion of the later factors can be excluded without a significant loss of data variance. In mathematical terms, the data matrix $\mathbf{D}$ can be reproduced, within some error $\mathbf{E}$,

$$\mathbf{D} = \mathbf{U}_n\mathbf{V}_n^{\mathrm{T}} + \mathbf{E} \tag{2}$$

where now the scores and loading matrices have been trimmed to include only the first n factors. By properly estimating n, the magnitude of the data set can be dramatically reduced. This has consequences for the ability to infer meaning to the data and for the computation time. The set of n factors retained is referred to as principal components or principal factors. One way to see how many factors [3,19] should be retained is by looking at the eigenvalues, since they give the amount of data variance retained by each factor. The eigenvalues or magnitude of variance for each factor decreases to a nearly steady state, which gives the magnitude of noise remaining. The number of factors to be retained, n, is determined when the addition of another factor does not contribute to any more information other than noise. Several ways to estimate the number of components have been

proposed [3,19,20]. This number of factors or principal components found by pure mathematical means is then related with the number of real sources of data variation. Special attention is paid to those principal components, which explain the larger portion of data variance. Components explaining little data variance (i.e. less than 1%) are not investigated and assumed to be mostly concerned with background and noise contributions.

PCA is designed to provide the best possible view of data variance. This view allows us to see the natural clustering in the data, identify outliers and find the reasons behind any observed pattern. From the amount of data variance retained by each factor and the contribution of the original variables to each factor, the chemical, biological or physical meaning of these factors can be assigned and the measurement noise can be estimated. Extremely useful tools in this context are the score and the loading plots derived directly from PCA analysis which, respectively, map samples and variables in the new vector space defined by the principal components. Score plots allow sample identification, and a check on whether they are typical or outliers, similar or dissimilar. Score plots allow us also to search for sample clusters (groupings). From loading plots the more important variables can be identified. Variables with large loadings close to each other and along the same straight line through the origin covary; and if they are at the same side of the origin they covary in a positive way, whereas if they lie in opposite sides, they are correlated negatively. Interpretation of clusters of samples in the score plot is simultaneously done by studying the corresponding loading plot.

Rotating the factors [3,19,21] to more closely align them with the directions of the original variables often enhances interpretation of the factors developed from a PCA model. Factor analysis (FA) utilizes factor rotation techniques that modify the loadings of contributing variables for each factor and provide new dimensional axes that may be more easily interpreted. The principal components or factor axes are ordered by decreasing variance and therefore, the first two or three axes provide most information about the data variance. In the varimax approach [21], the factor rotation attempts to maximize the variance of the loadings by sequentially rotating pairs of the original loadings. The varimax rotation is an orthogonal rotation of the factors that maximizes the variance of the squared factor loadings in a common factor, i.e. it tries to simplify the factor pattern. Different approaches have been described for the varimax rotation [21].

Target factor analysis (TFA) and target transformation analysis (TTFA) [22,23] are other non-orthogonal factor rotation procedures that realign the factor axes with axes that represent previously known profiles, e.g. source emission concentration profiles. TFA uses test vectors derived from existing knowledge of the relative composition of the actual sources. In TTFA, source profiles can be obtained by an iterative procedure from simple test vectors.

16.2.2.2 Hierarchical cluster analysis (HCA) and supervised classification methods (KNN and SIMCA)

Hierarchical cluster analysis [24] has been used to model the groupings of data into clusters showing similar attributes. The primary purpose of HCA was to present the data in a way that emphasizes the natural groupings in the data set. Distances between the samples (or variables) in a data set are calculated and compared. When the distances between samples are relatively small, the samples are likely to be similar, at least with respect to the

measurements taken. Dissimilar samples will have larger relative distances. The presentation of HCA analysis is usually in the form of a dendogram, making possible the visualization of clusters and the correlation among samples. Clusters are defined through distances (Euclidean), differences or similarities between two samples at each of the measured variables (concentration of the compounds). There are different ways to group the samples according to different distance measures and methods of linking samples.

One of the reasons why the acquisition of large amounts of data is so useful is because it allows the development of models to characterize and classify future samples in agreement with previously known or developed models. There are two types of measurable sample properties, either they are continuous valued properties or they will be discrete category assignments. If the property of interest has a continuous value a calibration model for the correlation between measured properties based on regression analysis techniques is possible (see below). But when the property of interest is a discrete category assignment, then classification modeling is the appropriate approach. Many classification models are based on the sample group assignment depending on its multivariate similarity with respect to other samples. Similarity techniques are based on the assumption that the more things resemble each other, i.e. the closer they lie together in the measurement space, the more likely their properties are of the same category. Two frequently used techniques are the K-nearest neighbour (KNN [25]) and the soft independent modeling of class analogy (SIMCA [26]). Although both are similarity-based techniques, their calculation approaches are different. KNN measures the Euclidean distance between the unknown sample and each of the known samples in the training set. The category assignment for the unknown is made by a plurality vote of the nearest neighbors. SIMCA, instead, models the location and distribution of a category in the measurement spaces by constructing a principal component representation of this distribution for each category. Class assignments for unknown samples are based on their proximity to the nearest category model. SIMCA models require having several samples per category, whereas KNN not. However, SIMCA has not only the ability to determine whether a sample belongs to any of the predefined categories, but also to determine that it does not belong to a certain class. This is also in contrast with KNN, which gives a class prediction regardless of whether the prediction is reasonable or not. SIMCA allows also placing confidence limits on any of the outcomes, because the decisions are made on the basis of statistical tests. Both KNN and SIMCA require that the training set samples have defined category (classes) assignments in the data. These assignments can be made from external knowledge, apart from the experimental measurement of the independent variables used to characterize the samples. If no external knowledge is available, it is possible to use HCA to assign the categories for the training analysis.

16.2.3 Data modeling

Many problems in environmental analysis can be solved by means of multivariate modeling methods. In fact, the purpose of many multivariate analyses will be to develop a model to predict a property of interest. Continuous properties are modeled and predicted by regression analysis [27]. The goal of regression analysis is to model the correlation between independently measured data and some property of the samples. Often, the

independent variables originate in spectroscopic or in chromatographic measurements, and these data would be used to predict the determination of some kind of bulk property or a concentration that cannot be measured directly either due to cost or the lack of a specific sensor. Multivariate least squares linear regression methods are particularly useful due to the increased precision attained from including multiple channels of data. Multivariate regression methods are distinguished from multiple regression methods in that the relation between a multivariate response (**D**) data set (and not an univariate response!) and multiple predictors (**C**, i.e. analyte concentrations) are modeled. The basic equation for multivariate regression is

$$\mathbf{D} = \mathbf{CB} + \mathbf{E} \tag{3}$$

where **D** is the $n \times m$ data set of responses, **C** the $n \times p$ data set of regressors, **B** the $p \times m$ matrix of regression coefficients and **E** the $n \times m$ error matrix. Each column of **D**, **B** and **E** corresponds to one of the m responses, each column of **C** and each row of **B** to one of the predictor variables and each row of **D**, **C**, and **E** to one of the n observations. In fact, multivariate regression analysis is essentially identical to a set of univariate regressions, where each column of **E** separately has a minimum sum of squares, i.e. when each univariate column of **D** is fitted by **C** in the least squares way. Consequently, the least squares minimization of **E** is obtained if each separate dependent variable is fitted by multiple regression on **C**. The solution for the regression parameters of previous equation is therefore

$$\mathbf{B} = (\mathbf{C}^T\mathbf{C})^{-1}\mathbf{C}^T\mathbf{D} \tag{4}$$

In the application of multivariate regression methods to analytical chemistry problems, in particular to multivariate calibration problems [28], two possible variants are used depending on how the model is formulated. These are classical least squares (CLS), where

$$\mathbf{D} = \mathbf{CB} + \mathbf{E_D} \tag{5}$$

or inverse least squares (ILS) where

$$\mathbf{C} = \mathbf{DB} + \mathbf{E_C} \tag{6}$$

Whereas in the CLS the analytical responses (spectra) are modeled as a function of the composition of the system, in ILS the opposite is established. This has important consequences. For instance, in many circumstances it is not possible to know explicitly all the variables in **C** affecting the observed variability in **D**, which makes the CLS model not practical. When environmental samples are analyzed using a spectrometric method, apart from the analyte many other uncalibrated components can cause the variability observed in the experimental spectra. This problem is solved when the inverse model is used (ILS), since then only that part of the variability related with the changes in composition for the analytes of interest is explicitly modeled during the calibration process. This means that the calibration of the system in the presence of unknown interferences is possible. However, ILS has other drawbacks, such as the requirement that the number of predictor variables (spectrum wavelengths) be equal or lower than the number of predicted values (samples). Another drawback of ILS is the need of inversion of matrix **D**, which is usually a highly collinear matrix. Some proposed alternatives involve selecting a reduced set of non-collinear variables in matrix **D** to avoid unstable estimations of the data matrix **C**. But

reducing the number of variables in **D** produces a loss of precision in the results because of the simultaneous loss of signal averaging properties in the estimations using a large number of variables in **D**.

16.2.3.1 Principal component analysis and partial least squares

Two more effective multivariate regression methods, both of which are based on factor analysis principles, are principal component regression (PCR) and partial least squares (PLS) regression [28]. In essence, both methods use an inverse least squares approach, like in ILS, but now the original variables are substituted by a subset of linear combinations (factors, components, latent variables) of them. In fact, PCR and PLS combine the advantage of using all the variables, avoiding noise (relegated to unused factors) and retaining the ILS independence of uncalibrated components.

The first step in PCR is the PCA matrix decomposition applied to the data matrix **D** (Eq. (1)). Once this data decomposition has been performed, and the major principal components selected, multivariate least squares regression of matrix **C** (inverse model) is performed using them

$$\mathbf{T} = \mathbf{C}\mathbf{V}^{\mathrm{T}} \tag{7}$$

where $\mathbf{V}^{\mathrm{T}}$ are the loadings PCA. This equation represents the projection of each variable in **C** onto the space spanned by the first principal components of **D**. In fact the PCR model coefficient matrix can be obtained by means of the equation

$$\mathbf{B} = \mathbf{V}^{\mathrm{T}}(\mathbf{T}^{\mathrm{T}}\mathbf{T})^{-1}\mathbf{T}^{\mathrm{T}}\mathbf{D} \tag{8}$$

The PCR approach has many advantages over the ILS approach. The reduction of the number of variables in matrix **D** is now accomplished, maintaining the maximum amount of information. The neglected minor components are supposed to contain mostly noise that is not relevant for the relation with the matrix **C**. As the obtained principal components are uncorrelated (orthogonal) matrix inversion is now not a problem at all and, as they explain maximum variance, the regression estimated parameters are more stable.

In PLSR a small number of components are also obtained, but now not only using the decomposition of matrix **D** as in PCA, but also simultaneously trying to find out first which of those components have better predictive capabilities for matrix **C**. The components are selected using another criterion than in PCR. PLSR has been introduced in the Chemometrics literature as an algorithm with the claim that it finds simultaneously important and correlated components of **D** and **C** matrices. Only recently have the PLS statistical and numerical properties been fully understood [29–31]. The PLS factors can be seen as modified principal components in which the correlation between the two data blocks (**D** and **C**) is improved at the cost of some decrease of the variance explained by each of them. In PLS, the component or factors are chosen to maximize the covariance between D and C datablocks.

These multivariate regression methods achieve their goals in basically the same manner, with one important difference: PCR uses steps similar to those used in PCA to decompose the data matrix of independent variables into principal components, then relates the calculated objects from the decomposition to the dependent variable(s). This relationship is reduced to a regression vector, which can be used subsequently to predict a value of the

dependent variable for a new, test samples. PLS approaches the decomposition of the independent variables in a similar way, but during the decomposition steps, information extracted from the independent variable matrix is passed to the dependent variable vector and vice versa. The results from PLS are also a regression vector, but one in which correlations between the independent block of data (the X block) and the dependent block (the Y block) are included. The goal of any regression analysis is to develop a calibration model, which correlates information in a set of measurements to some desired property (or properties). To fully test a model created in the calibration step requires a validation procedure. In general validation entails the application of a model to test samples for which the properties are already known. Thus, by comparing the predicted values to the known, we can establish a measure of reliability for the model.

The selection of the more appropriate number of factors to retain in the model is critical in developing a regression model. If an insufficient number of factors are retained, predictions will be unreliable because there will be important information left out of the model. On the other hand, if the regression model contains too many factors, prediction errors will increase because the model will contain noise, which will be transferred into predicted values. It is common for instance, to use structure in the eigenvalues as a means to deduce the proper number of factors or components, as in the use of the indicator function (IND) and F test [3,19], or to use cross-validation techniques [20]. As important as determination of the size of a regression model is the diagnosis and removal of outlier samples. Two of the diagnostics frequently used are the calculation of leverage and of studentized residuals. The first is a measure of the influence of a sample or a variable in the regression model. If a sample shows a profile that is different to those of the bulk of the training set, it will have an undue influence on the model, drawing the model closer to its location than justified. Prediction residuals are useful too in determining a model's reliability, especially once they have studentized, i.e. corrected by the root mean squared residual (RMSE) and the leverage [28].

PCR and especially PLS are very powerful tools for linear multivariate data modeling. Several robust PLS algorithms have been described [29–31] giving results that in some cases may allow a better interpretation of the data. When the relationships between multivariate measurements are non-linear ([32] and references herein), then the relationships between the independent variables (X matrix) and the dependent variables (Y matrix) are strongly non-linear, and non-linear modeling methods, such as artificial neural networks (ANN) [33,34], should be applied. The analysis of more complex environmental data structures by means of regression methods using higher order multiway regression methods has been also reported in the literature [35]. This is a field of increasing interest at present [36].

Model validation is usually performed with bootstrap, cross-validation or/and jackknife statistical approaches using resampling techniques or by training-evaluation experimental data set split. With these techniques, the goodness of prediction of a model and the estimation of the errors of the estimated parameters in terms of their bias and variance can be evaluated [1,2,5,20,28].

16.2.3.2 Time series analysis

Regression techniques can be also used for detection of trends in data series. Values in

the *x*-axis in time series analysis are mostly temporal data like day, month or year. In order to test a trend, a linearly increasing *x*-axis is needed. Explanatory variables may be any variables with a deterministic relationship to the time series. Very useful tools for analyzing time series are correlation techniques like autocorrelation, autoregression, partial autocorrelation and cross-correlation functions. The aim of correlation analysis is to compare one or more functions and to calculate their relationship with respect to a change of the lag of time or distance. Multivariate time series models are also available [37,38]. Multivariate correlation techniques enable inclusion of all interactions between multiple variables and the exclusion of the share of variance resulting from the variable noise. Powerful multivariate methods in time series analysis for describing and forecasting have been proposed, such as ARIMA modeling [39].

16.2.4 Detection of purest variables

Several methods [3,40,41] have been proposed to find the pure variables in a data set, i.e. those variables whose intensity only depend on the presence of a single species or component. In the SIMPLISMA approach [41] the estimation of the first pure variable is based on the evaluation of the relative standard deviation of the columns of a data matrix. A large relative standard deviation indicates a high purity of that column. In order to avoid that columns with a low mean intensity obtain a high purity value, the relative standard deviation is truncated by introducing a small offset value. Next pure variables are obtained from the largest determinants (largest dissimilarity) between the previously selected pure variables and all the possible combinations, considering all the other variables. The number of selected variables is equal to the rank of the analyzed data matrix, and in fact this technique can also be used for this purpose. Detection of pure variables allows also the initial estimation of species profiles in any of the two orders of measurement to be used in multivariate curve resolution (see below). Once the pure variables have been determined, the data set can be resolved into the pure components and their contributions in the original spectra. If for every component a pure variable is detected, the correct resolution can be achieved. However, pure variables in one of the two orders should exist in the data. Therefore, selectivity in one of the two orders for every species is needed. Use of derivatives to increase resolution has been also proposed [42].

16.2.5 Evolving factor analysis

Once the number of components is initially estimated by PCA or SVD, the changes and structure of an evolving data set arranged in a data matrix can be analyzed using Evolving Factor Analysis methods [43,44]. This approach provides an estimation of the regions or windows where the concentration of the different components is changing or evolving. Also, they provide an initial estimation of how these concentration profiles change along the experiment. The EFA method is based on the evaluation of the magnitude of the singular values (or of the eigenvalues) associated with all the submatrices of a matrix **D** built up by adding successively one by one all the rows of the original data matrix. The calculations are performed in two directions, forward (in the same direction of the experiment) starting with the two first spectra, and backwards (in the opposite direction of the experiment) starting with the two last spectra. In the forward direction, the detection of a new component is achieved by the upsurging of a new singular value; in the backward

direction, the disappearance of a component is detected also by the upsurging of a new singular value. In their graphical representation (EFA plots), singular values related with significant components become larger and clearly distinguished from the singular values associated with noise. Singular values related with the noise are smaller and they are at the bottom of the EFA plots. Interpreting the EFA plots and joining appropriately the lines corresponding to forward and backward singular values allows the estimation of the regions or windows of existence of each component and provides a first estimation of the *abstract* concentration profiles of the detected components. A more detailed description of the EFA plots can be found in previous works [43–45].

16.2.5.1 Evolving factor analysis with a fixed size moving window

A closely related and complementary method to EFA is the fixed size moving window evolving factor analysis (FSMWEFA) method [46]. In this case, the eigenvalues or singular values are calculated for submatrices of equal size moving in the same direction as the experiment is performed. The size of the matrix is chosen to be slightly higher than the suspected number of components simultaneously present (overlapping) along the experiment and kept constant. If this number is unknown, several sizes are attempted. The lower the sizes of the moving window, the better the local rank detection power; the larger the size of the window, the better the resolution power between similar components. As with EFA, the appearance of a new component is distinguished with the upsurging of a new singular value. The interpretation of the FSMWEFA plots allows the estimation of how many species coexist at the different stages of the experiment.

The study of the mathematical structure of the data matrix by the two evolving factor analysis-related techniques (EFA and FSMWEFA) yields a dynamic picture of the chemical process. The possibility to obtain this information from pure mathematical means can be important not only to understand the dynamic nature of a particular process, but also to outline the chemical and mathematical constrains to be applied in the resolution of the system by multivariate curve resolution (see Section 16.2.6).

16.2.6 Multivariate resolution

16.2.6.1 Multivariate resolution of two-way data

As has been pointed out in previous works [47,48], the source identification and apportionment from environmental data tables are problems similar to the species identification and resolution problems in spectrometric mixture analysis. In both cases, the goal of the analysis is the identification of the sources of data variance and the resolution of the profiles of these sources. The pure component spectra profiles resolved in mixture spectrochemical analysis are analogous to the chemical composition source profiles in environmental analysis, and the concentration are analogous to the contribution source profiles. In both cases an experimental data matrix (data table) is analyzed, which has a number of rows equals to the number of analyzed samples or measured spectra and with a number of columns equals to the number of measured variables, wavelengths or different analyzed chemical compounds. In both cases it is assumed that this data matrix is bilinear, i.e. that it follows a linear model and it can be decomposed in the sum of a reduced number of individual (rank one) contributions (chemical species in spectrochemical analysis or envir-

onmental sources in environmental analysis). This analogy shows that methods like curve resolution [49–51] which were initially developed for the analysis of chemical processes monitored spectroscopically can be also applied for the resolution of environmental sources from environmental data sets as those of interest here.

Multivariate curve resolution (MCR) [52–54] is a chemometric method in the factor analysis (FA) family of techniques [3]. Its principal goals are the isolation, resolution and, eventually, quantitation of the sources of variation in a particular data set. An outstanding feature of this technique is that no a priori assumption about the contribution of the different factors in the global response is necessary. In previous works [47–49], MCR has been successfully applied to the study of different type of evolutionary chemical and analytical processes. From detection of purest variables or local rank analysis, initial estimations of species or source profiles are available, from which a constrained alternating least-squares (ALS) optimization is applied to recover physically meaningful profiles of the individual species or sources, which best explain the observed data variance. As was previously mentioned, this optimization is based on the assumption that the data matrix is bilinear, i.e. that it can be decomposed in the product of two matrices

$$\mathbf{D}(NR, NC) = \mathbf{C}(NR, N)\mathbf{S}^{\mathrm{T}}(N, NC) + \mathbf{E}(NR, NC) \tag{9}$$

In this equation $\mathbf{C}$ is the matrix whose N columns describe how the N identified environmental sources or chemical species change between samples or during the process (contribution or concentration profiles); the number of rows of $\mathbf{D}$ and $\mathbf{C}$ matrices, NR, are equal to the number of samples/spectra used in the analysis. $\mathbf{S}^{\mathrm{T}}$ is the matrix whose N rows describe how are the detected sources or species (composition or pure spectra profiles); the number of columns of $\mathbf{D}$ and $\mathbf{S}^{\mathrm{T}}$, NC, are equal to the number of variables measured, wavelengths or chemical constituents analyzed. Equations (1) and (9) show two possible ways of decomposing the same data matrix. In fact, owing to the rotational and intensity factor analysis decomposition ambiguities, there are an infinite number of possible mathematical decompositions of a data matrix or two-way data set that reproduce the original data matrix equally well. All these possible mathematical decompositions, however, do not have the same physical meaning (see below), and the goal of MCR methods is to find only those solutions which satisfy a set of constraints postulated from the physical and chemical knowledge of the studied system. Finally, matrix $\mathbf{E}$ has the residual data variance not modeled by the N detected sources and it has the same dimensions as $\mathbf{D}$.

The mathematical problem stated by the model in Eq. (9) can be summarized in the following way. Given data matrix $\mathbf{D}$, find the least squares source optimal contribution or species concentration profiles defined in matrices $\mathbf{C}$ and the least squares optimal source composition or species pure spectra profiles defined in matrix $\mathbf{S}^{\mathrm{T}}$. First, the number of significant contributions to the whole data variance, N, must be estimated. In the analysis of environmental data tables, the analysis will obviously be focussed on the major and distinct sources of data variance and not on the small contributions coming from multiple minor sources of data variation. Hence, for a model with a particular number of contributions, N, the residual matrices, $\mathbf{E}$, will still have a significant percentage of unexplained data variance coming from multiple minor unknown source contributions. This situation is clearly different to the situation usually encountered in the spectrochemical analysis of mixtures where most of the data variance should be explained by the selected number of

components. In MCR, the selection of the number of source contributions, N, is initially estimated simply from the size of the eigenvalues of the experimental data matrix **D**. A simple plot of them gives a quick indication of the probable number of major sources. Moreover, principal component analysis (PCA [8]) is also applied to this matrix to see what is the percentage of explained data variance for a particular number of components, defined by

$$\%\mathrm{var} = \frac{\sum\limits_{i,j}(d_{i,j}^{e} - d_{i,j}^{c})^{2}}{\sum\limits_{i,j}d_{i,j}^{e2}} \times 100 \tag{10}$$

where $d_{i,j}^{e}$ are the experimental data values and $d_{i,j}^{c}$ are the corresponding calculated values using N components in the PCA model. Once a first estimation of the number of components is available, the matrix decomposition using Eq. (9) is not unique since there is rotational and scale freedom in the unconstrained solutions. This means that there are an infinite number of possible solutions if no constraints are set during the linear data matrix decomposition formulated in these equations. The conditions and constraints under which it is possible to recover the true solutions of Eq. (9) for **C** and $\mathbf{S}^{T}$ have been studied elsewhere [53,55]. When these conditions and the appropriate set of constraints are applied, the obtained solutions are very close or eventually equal to the true ones. A short summary of the optimization procedure proposed to solve iteratively Eq. (9) is given.

When an initial estimation of the individual spectra is available, the best least squares unconstrained solution of the concentration profiles is estimated from

$$\mathbf{C} = \mathbf{D}(\mathbf{S}^{T})^{+} \tag{11}$$

where $(\mathbf{S}^{T})^{+}$ is the pseudoinverse [56] of the $\mathbf{S}^{T}$ matrix.

If, in contrast, an initial estimation of the concentration profiles is available, the best unconstrained least squares estimation of the spectroscopic contributions is estimated from

$$\mathbf{S}^{T} = \mathbf{C}^{+}\mathbf{D} \tag{12}$$

where $\mathbf{C}^{+}$ is now the pseudoinverse of **C** matrix.

The least squares solutions obtained in this way are optimal least squares solutions, which probably will not be optimum from a chemical point of view. For instance, they can have negative concentrations. Therefore a least squares optimization procedure is used resolving iteratively the two equations previously given and constraining, at each stage of the iterative optimization, the solutions to be non-negative [57,58]

$$\mathbf{C}_{\mathbf{aug}} > 0 \text{ and } \mathbf{S}^{T} > 0 \tag{13}$$

Other constraints which can be implemented during the ALS optimization are closure (sum of the values of all contributions per sample is equal to a known constant value), unimodality (some profiles are known to have unimodal peak or cumulative shapes [59,60]) and equality constraints (some values in the profiles are already known, e.g. in some cases some values are known to be zero because of selectivity or the values of some profile are known a priori). Most of these constraints however, cannot be applied in general in the analysis of environmental data tables, where in fact the more useful constraint is

non-negativity. Details about the implementation of the MCR–ALS method have been described elsewhere for different types of chemical data [61–65].

16.2.6.2 Multivariate resolution of three-way data

An interesting aspect of many environmental data sets is that they have a three-way data structure [33–36]. This means that many environmental data sets can be ordered using three ways or orders of measurement, as for instance measured variables on each sample (what analyte concentrations are measured), where these samples were measured and when these samples were measured. As there are three ways of measurement, there will be corresponding three-way profiles for each of the resolved components: the loadings (pure spectra or composition profiles), the time scores (time contributions, distributions or profiles); and the geographical site scores (site contributions, distribution or profiles). Also, in the case of spectrochemical analysis of mixtures, different experiments or determinations can be performed under different experimental conditions, giving each experiment a single data matrix D_i, and as before, three or more ways of measurement are obtained (see Fig. 16.2). Multivariate curve resolution can be easily applied to the simultaneous analysis of several individual data matrices [52–54]. In analogy to the analysis of a single data matrix, the new column-wise augmented matrices can be decomposed in the product of two matrices

$$
\begin{pmatrix} D_1 \\ D_2 \\ \cdots \\ \cdots \\ D_{72} \end{pmatrix}
\begin{pmatrix} C_1 \\ C_2 \\ \cdots \\ \cdots \\ C_{72} \end{pmatrix} S^T +
\begin{pmatrix} E_1 \\ E_2 \\ \cdots \\ \cdots \\ E_{72} \end{pmatrix}
\tag{14}
$$

$$
\mathbf{D_{aug}} = \mathbf{C_{aug}} \mathbf{S}^T + \mathbf{E_{aug}}
\tag{15}
$$

where $\mathbf{D_{aug}}$ and $\mathbf{C_{aug}}$ are respectively, the column-wise augmented data and source contribution or species concentration matrices, and $\mathbf{S}^T$ is the non-augmented source composition or species spectra matrix. In order to have a meaningful column-wise data augmentation, the common sources (species) in all the individual matrices D_i should be equal, i.e. they should have the same composition or pure spectra profile $\mathbf{S}^T$, although their contribution (concentration) profiles $\mathbf{C}$ may change between different data matrices. This is the same as to say that in the data model given in Eqs. (14) and (15), data are not necessarily trilinear (see below), although data are still bilinear and share one of the two orders of measurement between the different matrices D_i simultaneously analyzed.

The alternating least squares multivariate curve resolution method previously described for individual data matrices can be easily extended to the analysis of the column-wise augmented data matrices. The equations to be solved now are

$$
\mathbf{C_{aug}} = \mathbf{D_{aug}} (\mathbf{S}^T)^+
\tag{16}
$$

and

$$\mathbf{S}^{\mathrm{T}} = (\mathbf{C}_{\mathrm{aug}})^{+} \mathbf{D}_{\mathrm{aug}} \tag{17}$$

where $(\mathbf{S}^{\mathrm{T}})^{+}$ and $(\mathbf{C}_{\mathrm{aug}})^{+}$ are the least squares estimations of the pseudoinverse [56] of $\mathbf{S}^{\mathrm{T}}$ and $\mathbf{C}_{\mathrm{aug}}$ matrices. These two equations are solved iteratively under the non-negativity constraints [57,58]:

$$\mathbf{C}_{\mathrm{aug}} > \mathbf{0} \text{ and } \mathbf{S}^{\mathrm{T}} > \mathbf{0} \tag{18}$$

Eventually other constraints like unimodality [59,60], closure or others [52–54] can be also applied. In order to start the iterative process, initial estimations are needed either for $\mathbf{S}^{\mathrm{T}}$ or for $\mathbf{C}_{\mathrm{aug}}$. For instance, initial estimates for $\mathbf{S}^{\mathrm{T}}$ or for $\mathbf{C}_{\mathrm{aug}}$ are obtained from detection of purest variables or samples [40–42] or from evolving factor analysis methods [43–45].

Once the ALS iterative matrix decomposition converges to a minimum, the percentage of explained variance is also calculated using the same expression as that used for PCA, Eq. (10), substituting the $d_{i,j}^{c}$ values for the corresponding calculated values using the N components obtained by the ALS matrix decomposition. The profiles obtained for $\mathbf{C}_{\mathrm{aug}}$ and $\mathbf{S}^{\mathrm{T}}$ are now directly interpretable since they refer to physical meaningful values. $\mathbf{S}^{\mathrm{T}}$ gives the source compositions (relative concentrations of the analytes in the sources (species spectra profiles in spectrochemical analysis) and $\mathbf{C}_{\mathrm{aug}}$ gives the source contributions (species concentration profiles in spectrochemical analysis). The conditions under which the ALS matrix decomposition using Eqs. (11)–(13) depend on data selectivity (unique source compositions or source contributions) and on local rank conditions [53,55]. The conditions for unique solutions of two-way data decompositions have been analyzed in detail by several authors for chromatographic and spectrometric mixture analysis data and they can be extended to environmental data.

Whereas the matrix of source composition $\mathbf{S}^{\mathrm{T}}$ is directly interpretable giving the analyte composition of each of the resolved environmental sources, the matrix of the source contributions or distributions $\mathbf{C}_{\mathrm{aug}}$ needs some rearrangement. The reason for this is that this matrix has two orders of information (i.e. temporal and spatial, or in spectrochemical analysis of mixtures, changes of concentration within one experiment and relative concentrations between experiments). From how the different data matrices were joined to build the augmented matrix $\mathbf{D}_{\mathrm{aug}}$, the resolved profiles in $\mathbf{C}_{\mathrm{aug}}$ can be unfolded and the proper physical meaning recovered. In the case of three-way data, the alternating least squares algorithm previously described for the decomposition of augmented data matrices can be easily adapted to constrain the solutions to be trilinear like in PARAFAC models, ALS based methods [66] or non-ALS non-linear optimization based methods [67]. In all these cases the individual contribution profiles of every component in each data matrix included in the augmented matrix have exactly the same shape. This is the same as to consider that instead of a matrix decomposition following Eq. (9) and Fig. 16.3, the three-way trilinear decomposition is obtained using the following model:

$$D_{ijk} = \sum_{n=1}^{N} t_{in} c_{jn} s_{kn} + e_{ijk} \tag{19}$$

The algorithm used by the MCR method to achieve that the solutions obtained in the ALS decomposition of augmented matrices be trilinear (see Fig. 16.3) has been described elsewhere [62,68]. In many circumstances, it is not reasonable to assume that three-way
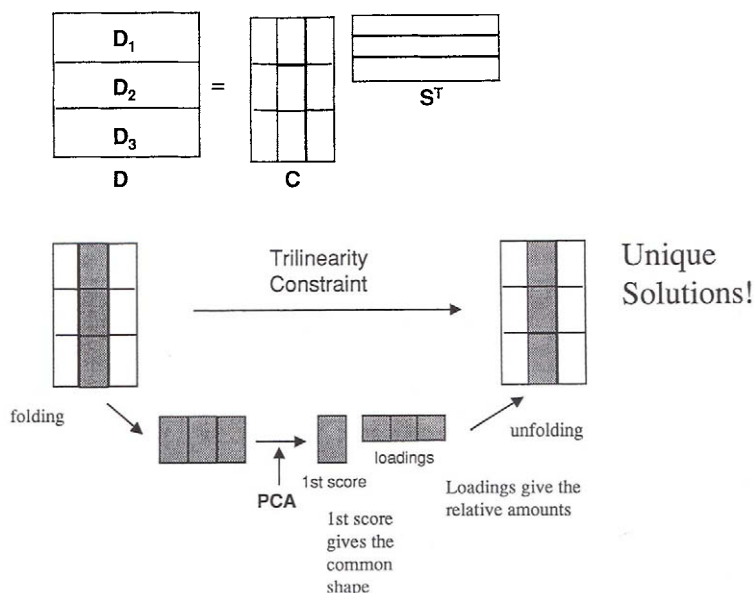
Fig. 16.3. Graphical explanation of the trilinearity constraint.

data fully agrees with a trilinear model following Eq. (19), although they are still bilinear and follow the model given by Eqs. (14) and (15). Therefore, the results obtained assuming trilinearity or not should be always compared. When the assumption of trilinearity implies a significant decrease in the percentage of explained variance of the experimental data [10] compared with that obtained when no-trilinearity is assumed, the assumption of trilinearity is at least questionable if not totally unreasonable. In the MCR–ALS method, trilinearity can be imposed for only some and not all the source profiles, which can be a clear advantage in some difficult cases.

16.3 EXAMPLES OF APPLICATION

To illustrate the use of some of the chemometric techniques previously summarized, the following examples of our previous investigations in this field are briefly described.

1. Characterization of sedimentary organic contaminants and molecular markers in the northwestern Mediterranean Sea by exploratory data analysis.
2. Resolution of herbicides and metabolites sources in US Midwest water reservoirs.
3. Resolution of pesticide degradation products by means of spectrometric methods.
4. Resolution of chromatographic unresolved mixtures of pesticides.

16.3.1 Characterization of sedimentary organic contaminants and molecular markers in the northwestern Mediterranean Sea by exploratory data analysis [69]

The objectives of this study were threefold.

(i)   the classification of the different depositional zones of study (NW Mediterranean Sea) according to the source of organic compounds occurring in surface sediments;
(ii) the classification of a large variety of organic compounds according to their different origin; and
(iii) the apportionment of input sources in the different samples.

For this purpose, a data set containing concentrations of biogenic (i.e. odd carbon numbered n-alkanes, sterols) and individual anthropogenic compounds (i.e. even carbon numbered n-alkanes, PCBs, PAHs, DDTs, etc.) from surface sediments were collected from the NW Mediterranean Sea. The initial exploratory data analysis was carried out including the entire geographical area: a data set containing 31 samples and 59 compounds (n-alkanes, PCBs, PAHs, chlorinated pesticides) (1829 values) was considered in this study. A second data set excluding the Barcelona and Rhône prodelta samples was built including 22 samples and 96 compounds (n-alkanes, PCBs, PAHs, sulfur containing PAHs, chlorinated pesticides, sterols) (2112 values). Experimental details are given in refs. [70,71]. The data analysis consisted in an exploratory study by principal component analysis (PCA), followed by hierarchical cluster analyses (HCA) and mixture resolution of apportionment sources using multivariate curve resolution alternating least squares (MCR–ALS).

Experimental data were arranged in a single data matrix, **D**, with as many rows as samples analyzed and as many columns as variables or analyte concentrations measured. The columns of the original data matrix (concentrations of a particular component in the different samples) were scaled to the same units dividing each element of the matrix by the standard deviation of its column. The new data matrix was not centered in order to not miss the reference information about the data center (like in apportionment studies [47,48]). An initial exploratory data PCA analysis was carried out including the entire geographical area and considering the following individual molecular markers: n-alkanes, PAHs, PCBs and DDTs. The first two principal components (PCs) accounted for 84.2 % of the total variance. Almost all variables, except op'-DDD, op'-DDE, benzo[ghi]fluoranthene and perylene, contributed to the first PC (Fig. 16.4), which accounted for 75.4% of the data variance and they were positively correlated. These results indicated that all pollution inputs were evenly distributed in the area of study (i.e. urban and industrial), probably due to their geographical proximity (Rhône river, gulf of Lion and Barcelona urban area). Furthermore, the lower contribution of op'-DDD and op'-DDE to the first variable could be attributable to the metabolically derived origin from o,p'-DDT of these compounds. Similarly, perylene, which has a dual origin – either diagenetic or pyrolytic – had a smaller contribution to the first PC in comparison with the remaining pyrolytic PAHs. Benzo[ghi]fluoranthene had also a small contribution to the first PC, indicating a singular environmental pathway for this compound. The second PC (Fig. 16.4) accounted for 8.8% of the total variance attributable to a positive contribution of some of the 3–5 aromatic ring PAHs (i.e. benzofluoranthene isomers, benzopyrenes, perylene, fluoranthene, pyrene, anthracene) mostly of pyrolytic origin, and to the transformation products of DDT (i.e. op'-DDE and o,p'-DDD). Parent pesticides were negatively correlated according to this PC (op'-DDT). Retene, usually associated either to wood combustion or diagenetic origin, was negatively correlated with pyrolytic PAHs. By plotting the scores of the first two PCs, the areas of study can be grouped in three
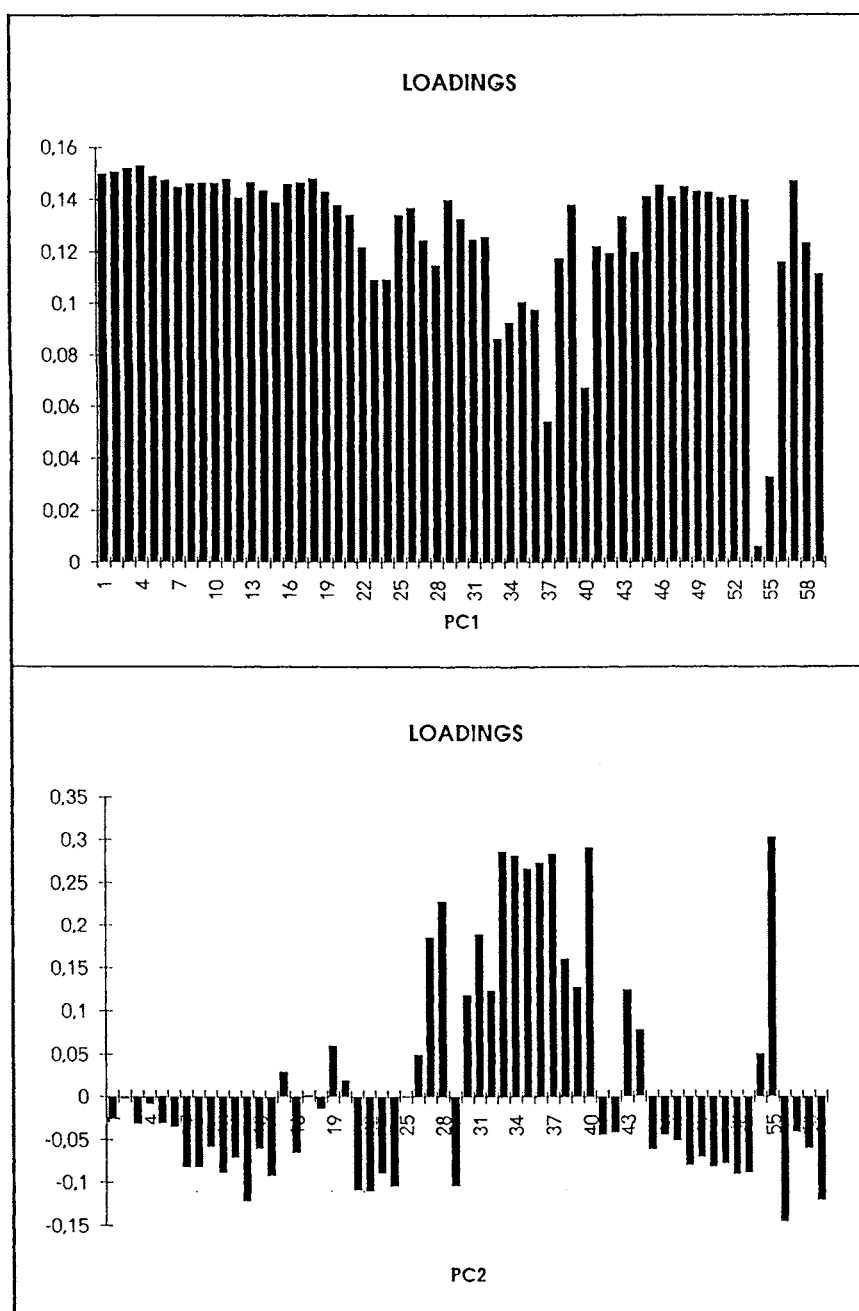
Fig. 16.4. PCA loading plots for the first data set (NW Mediterranean study, reproduced from [69]).

clusters (Fig. 16.5). A first cluster has a single sample located off-shore Barcelona which exhibited the highest contribution of PC-2. A second cluster is formed by the samples located in front of Rhône prodelta characterized by the highest values of PC-1. And a dense third cluster is detected containing the remaining samples, which have the lowest values for both PCs. These results are consistent with (i) the higher pollution levels found off shore Barcelona and the Rhône prodelta and (ii) the pollution of these areas is associated with different sources. Indeed, the higher contribution of PC-2 in the offshore Barcelona sample is accounted for by the o,p'-DDE associated with local sources of pollution and the pyrolytic PAHs coming from mobile sources [18].

In order to get further insight into the sources of the remaining samples, the offshore Barcelona and Rhône prodelta samples were dropped from the data matrix. Thus a new data subset containing the former variables and sterols was built up. The resultant new submatrix had 96 variables (compounds) and 22 samples. In this case, the first three PCs accounted for 76.6% of the total data variance (see Table 16.1). The positive contribution to the first PC (Fig. 16.6) is accounted now by compounds of biogenic or anthropogenic origin coming from land-based sources, i.e. from higher plants (n-C_{31}, n-C_{33}, and 24-ethylcholest-5-en-3β-ol), fossil sources of hydrocarbons (n-C_{17} to n-C_{29}, pristane, phytane, UCM), wood combustion (retene), diagenetic origin (perylene), coal mining (1-naphthothiophene), industrial origin (PCBs) and pesticides (lindane, DDTs and its metabolites). The most probable route of transport of all these compounds could be associated with continental runoff and/or river transport. A negative correlated contribution was evident for the rest of the PAHs (Fig. 16.6) and higher molecular weight n-
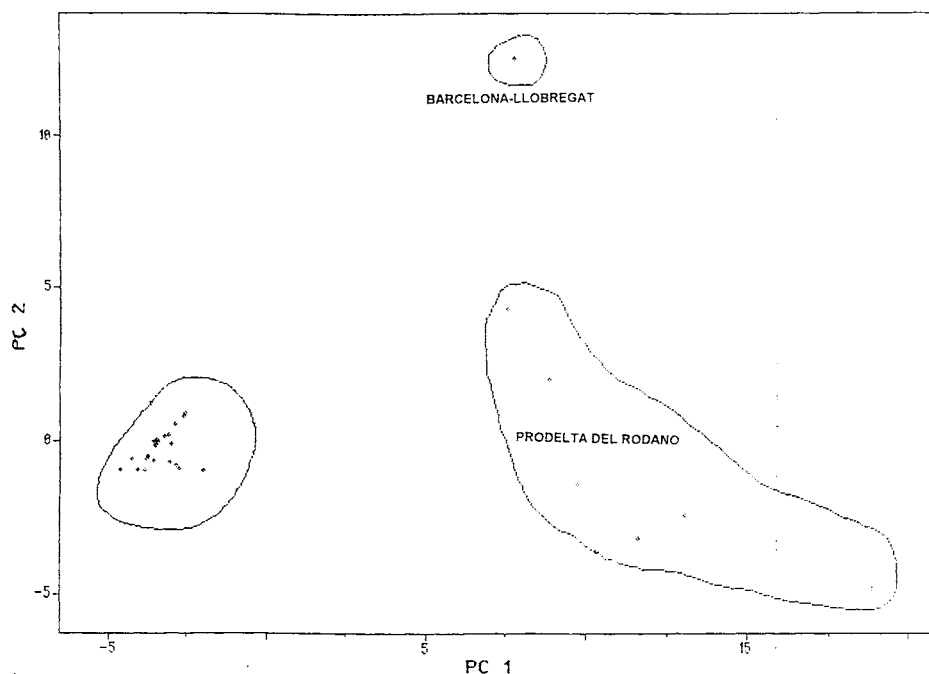
Fig. 16.5. PCA score plots for the first data set (NW Mediterranean study, reproduced from [69]).

TABLE 16.1

PERCENTAGE OF VARIANCE EXPLAINED BY PCA AND MCR–ALS (NW MEDITERRANEAN STUDY [69])

Component	PCA (autoescaled data)		PCA (standardized data)		MCR–ALS (standardized data)	
	%	% cum	%	% cum	%	% cum
1st	41.4	41.4	53.6	53.6	19.1	19.1
2nd	26.8	68.2	14.9	68.5	27.0	46.2
3rd	8.4	76.6	4.8	73.3	26.8	73.0

alkanes (n-C_{34-39}). While the former are associated to combustion processes and atmospheric transport the latter can be associated with tanker ballast operations carried out in the open sea. This first PC represents 41.4 % of the total variance (Table 16.1). From the score plots shown in Fig. 16.7, it is evident that the Ebro prodelta samples exhibited the highest contribution to the PC-1 value. The second PC represented 26.8 % of the total variance and shows the contribution of n-alkanes, pyrolytic PAHs and to a lesser extent PCBs, DDTs and sterols. The higher contribution of this PC in the Gulf of Lion samples is consistent with a predominant contribution of atmospheric deposition for this PC. On the other hand, samples located in the deep basin formed another cluster, which is characterized by intermediate values of both PCs. This grouping is consistent with the contribution of both atmospheric and adjective transport from the continental shelf. Scores plot PC-1 vs. PC-2 showed clearly three clusters of samples identified as Ebro Prodelta, Central Zone and Gulf of Lion. Loading plot (Fig. 16.7) PC-1 vs. PC-2 shows two groups of contributions separated by PC-1, those mostly constituted by n-alkanes and PAHs and those contributed by other n-alkanes, PCBs and DDTs. From comparison of scores and loadings plots it is concluded that Ebro Prodelta samples are mostly distinguished by the contribution of n-alkanes, PCBs, DDTs, pristane and that distinction between Gulf of Lion samples and Central Zone is accomplished by different content of n-alkanes and PAHs.

Hierarchical cluster analysis results are shown in Fig. 16.8. The linking method that gave results more similar to those obtained by PCA was the Incremental link method [1,10]. This method uses a sum of squares approach for calculating the nearest cluster. Other linking methods, like the single link method, the centroid link method or the complete link method, among others [1,10], produced slightly different groups. Three sample groups could be modeled from data similarities in agreement with the three clusters found by PCA. Two samples, which were located at the bottom of the slope of the Ebro Prodelta, were grouped with the Gulf of Lion cluster. These two samples exhibited a high composition similarity with the remaining samples from the Gulf of Lion. Although no definitive explanation could be given for this, these results can be explained by the fact that the slope of the Ebro Prodelta zone is characterized by large sedimentation rates due to the advective transport from eroded sediments coming from the slope and continental shelf. Furthermore, another sample collected in the edge of Rhône prodelta exhibited a higher similarity by HCA with the deep basin samples than with the Gulf of Lion. Taking into consideration that the prevalent sea current is NW–SE trended [36], the
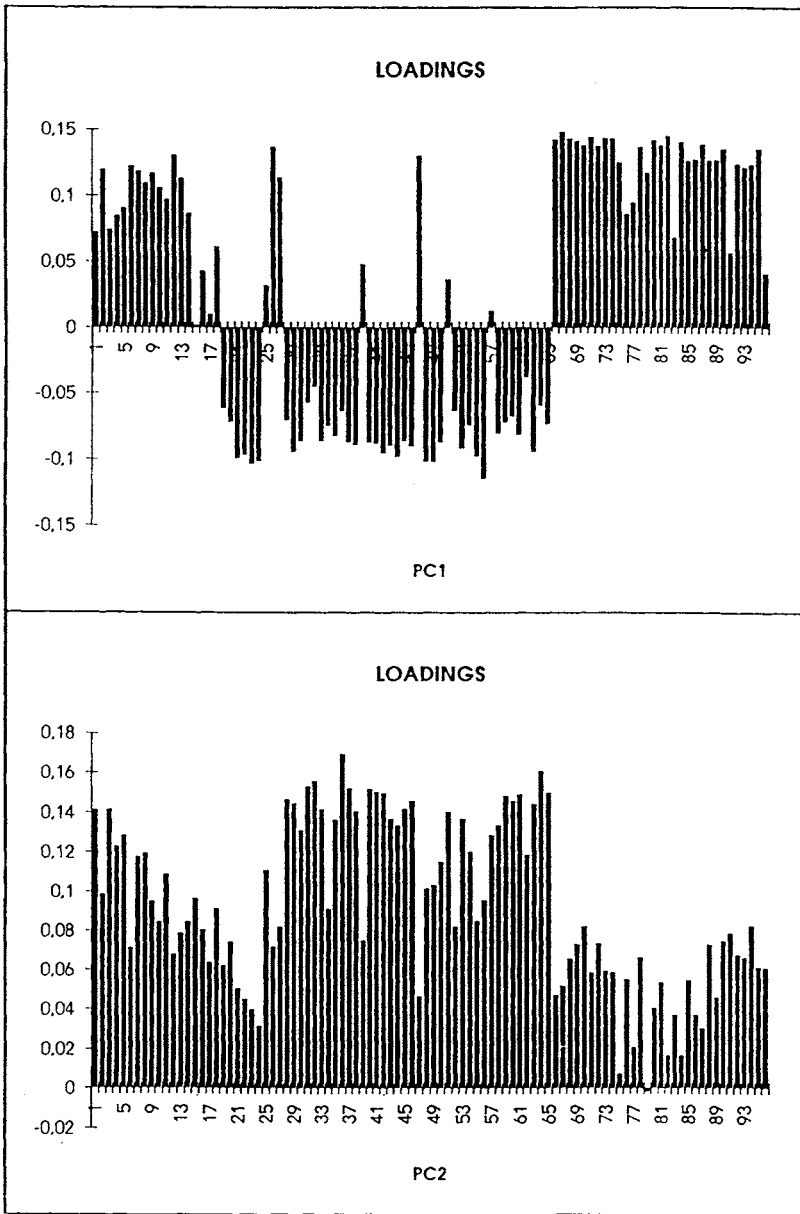
Fig. 16.6. PCA loading plots for the second data subset (NW Mediterranean study, reproduced from [69]).

Rhône plume could affect this sample. Therefore, both river transport and atmospheric deposition could be the main routes of organic matter transport into this region. Consequently, its grouping is closely related to the deep basin samples where both inputs are apparent.
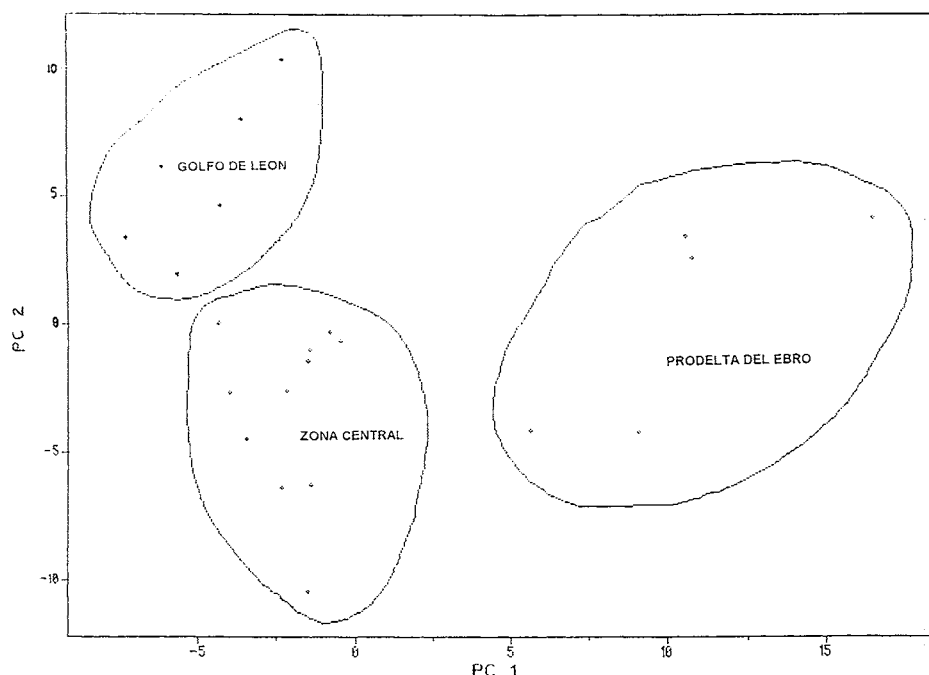
Fig. 16.7. PCA score plots for the first data subset (NW Mediterranean study, reproduced from [69]).

In the study of the reduced data matrix (without Barcelona and Rhône samples and incorporating sterol variables) three main components were initially considered for the MCR–ALS analysis, in accordance with previous results by PCA. The amount of experimental data variance explained by three components (data matrix was column scaled but not centered) were around 73% (Table 16.1), which was considered satisfactory for this type of studies. The initial basic assumption was that these three contributions could be associated with three different specific environmental sources, and that the remaining data variance (27%) was caused by background contributions not related with significant environmental sources. The initial estimation of the composition profiles of these three proposed environmental sources was made from the purest samples detected from the data matrix (see above and [40,41]). These three initial profiles correspond to the concentration compositions of a characteristic sample from the Ebro Delta zone, a sample of the slope and a sample from the deep basin. The application of the multivariate resolution method previously described using these three initial estimations of the source composition gave an optimum set of composition and contribution profiles for each of these three identified environmental sources (Figs. 16.9 and 16.10). The amount of experimental data variance finally explained using the proposed ALS method was similar to that obtained with the PCA method (ca. 73%) which confirms that the same amount of data variance can be explained using both methods. However, the interpretability of the ALS recovered profiles is easier to that obtained by PCA. In Fig. 16.9, the normalized concentration compositions of the three-resolved source profiles of matrix S^T are given. The first resolved source composition profile (Fig. 16.9) had high concentration inputs, mostly of PAHs, which
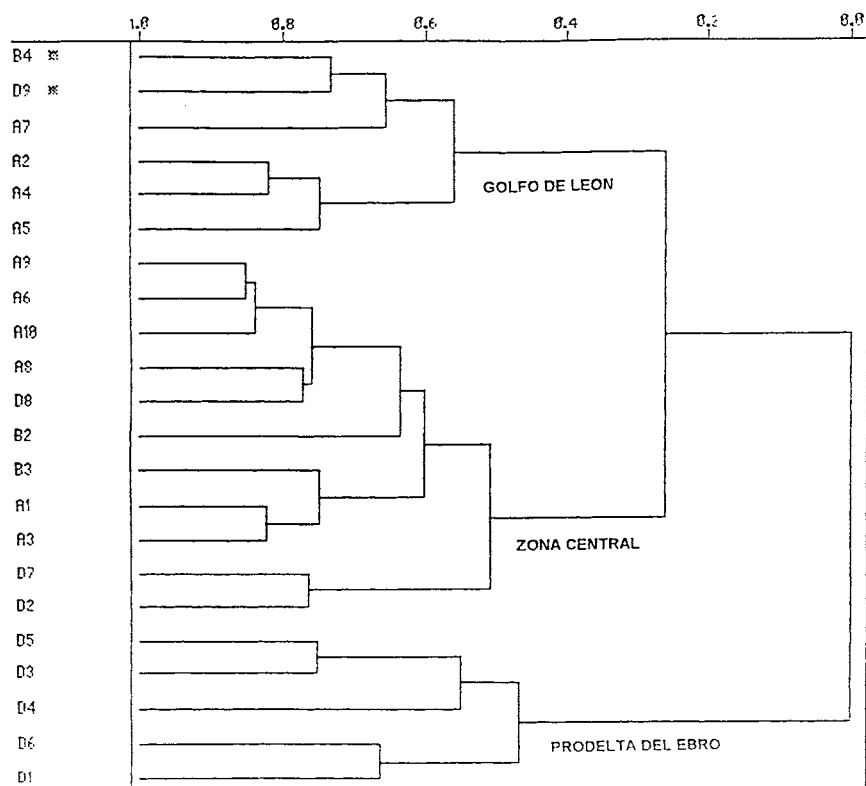
Fig. 16.8. HCA dendogram obtained for the second data subset (NW Mediterranean study, reproduced from [69]).

are assumed to be of anthropogenic origin. Perylene, which has a dual origin (either diagenetic or pyrolytic), made a small contribution to the first component. UCM and some n-alkanes and sterols also had high concentration input. The second resolved source composition profile (Fig. 16.9) had very low inputs of all of these components but it had higher concentrations for n-alkanes, pristane, phytane, perylene, PCBs, pesticides and sterols, which are assumed to be of continental river origin (Ebro river). Finally the third resolved source composition profile (Fig. 16.9) had only some high n-alkane concentrations and very low concentrations from the other components. It would correspond to an unspecific background origin associated with long-range transport of contaminants subjected to degradation and transformation processes during transport. The contribution profiles of the three resolved sources according to the sampling site are shown in Fig. 16.10. The higher contribution of the first resolved source profile was found in the Gulf of Lion samples. The higher contribution of the second resolved source profile was found in the Ebro area and the third source in the open sea sampling sites. All these results are in agreement with those previously found by PCA and HCA methods, which on one side validates the former results but on the other hand reveals the capability of the proposed
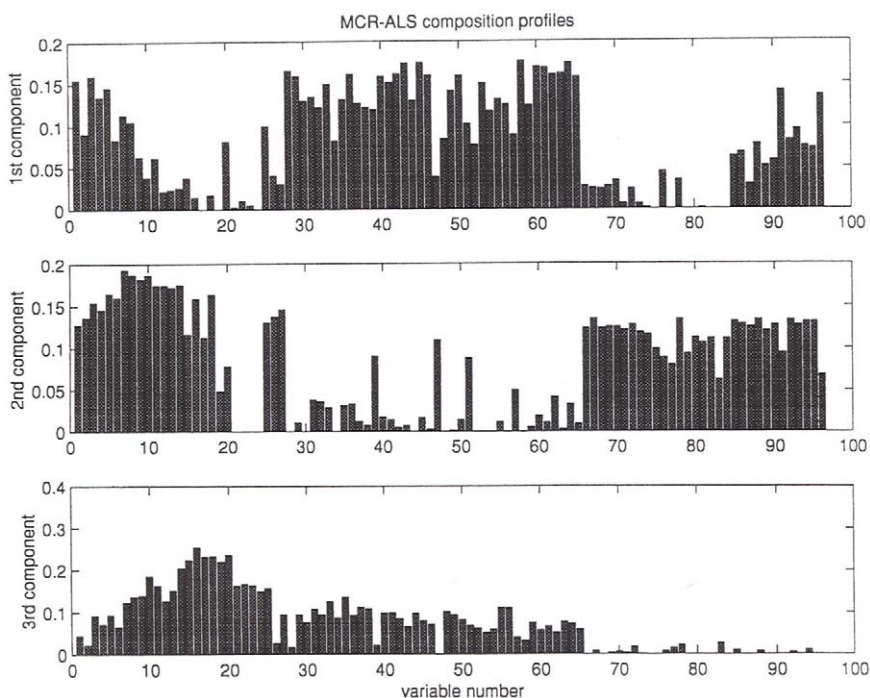
Fig. 16.9. MCR–ALS contribution profiles (matrix **C**) obtained for the second data subset (NW Mediterranean study, reproduced from [69]).

MCR–ALS approach achieving multiple source apportionment as is the case in most of environmental studies.

16.3.2 Resolution of herbicides and metabolites sources in US Midwest water reservoirs [72]

In this case the study area comprised about 720 000 m^2 land, from 11 US States (Illinois, Indiana, Iowa, Kansas, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin), that drain to the Ohio, upper Mississippi and lower Missouri Rivers. Herbicide sample bottles were analyzed at the USGS laboratory in Lawrence, Kansas. Eleven herbicides, two atrazine metabolites and three cyanazine metabolites were analyzed using GC/MS. The analysis included alachlor, ametryn, atrazine, cyanazine, metolachlor, metribuzin, prometon, prometry, propazine, simazine and terbutyrin as well as two atrazine metabolites, deethylatrazine and desisopropylatrazine, and three cyanazine metabolites, cianazine amide, deethylcyanazine and deethycyanazine amide. In addition the ethane sulfonic acid (ESA) metabolite of alachlor was isolated by solid-phase extraction (SPE) and analyzed by ELISA. Further details about analytical determinations, quality assurance and analytical results are given elsewhere [73,74]. Outflow from each reservoir was sampled eight times (approximately bimonthly) from April 1992 through September 1993. The timing and frequency of these samples made it
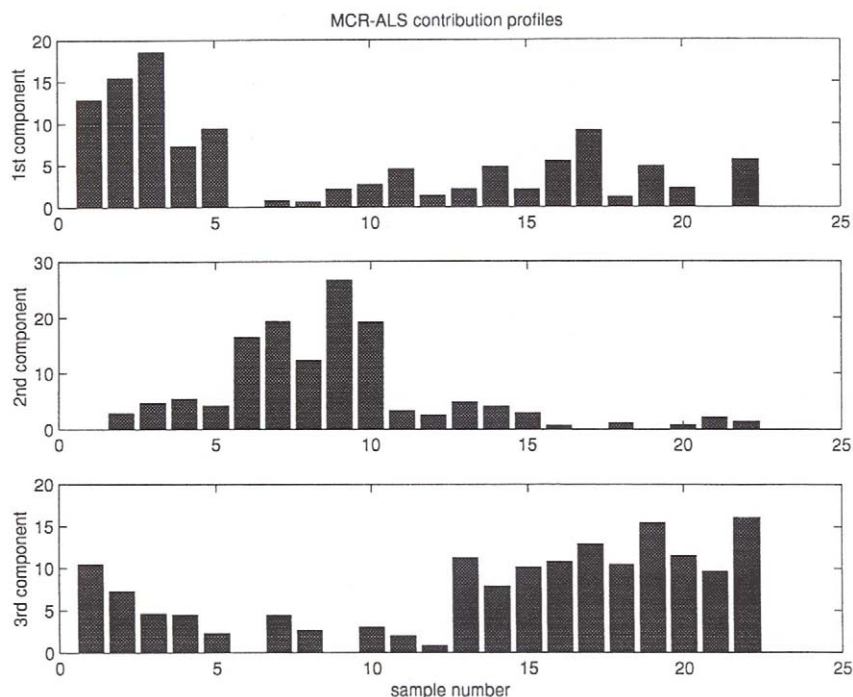
Fig. 16.10. MCR–ALS composition profiles (matrix S^T) obtained for the second data subset (NW Mediterranean study, reproduced from [69]).

possible to determine approximately when maximum and minimum concentrations of herbicides occurred in the reservoir outflow.

The whole data set had 9120 entries; it consisted in the concentration (μg/l) of 15 herbicides and herbicide metabolites in samples from 76 water reservoirs at different locations of the US measured during eight time periods, from April 1992 through September 1993. In Fig. 16.11 a summary of the concentrations measured for each of the analytes at the different reservoirs and collection times is given. As there were three different identification indexes for each concentration value, (analyte, reservoir and collection time), the experimental data could be ordered in a three-way data structure (data cube, see Fig. 16.2). In this work, the preferred data arrangement was the one, which considered this data cube unfolded in a column-wise augmented data matrix with 608 row-samples (76 reservoirs × 8 sampling times) and 15 column variables (see Fig. 16.2). This data arrangement provided a faster insight in the primary goal of this work, which was the determination of the occurrence and distribution of the analytes in outflow from selected reservoirs in the upper USA Midwest. Two additional problems considered were the presence of a large number of missing values and of values below the limit of detection.

From the 9120 entries in data matrix D_{aug}, 284 were missing (3.1% of the total). The distribution of these missing values was not uniform. For some variables, like variables 8, 9 and 10, i.e cyanazine derivatives, the number of missing values was very high, 94 missing values from 608 values for these three variables (approximately 15% of the
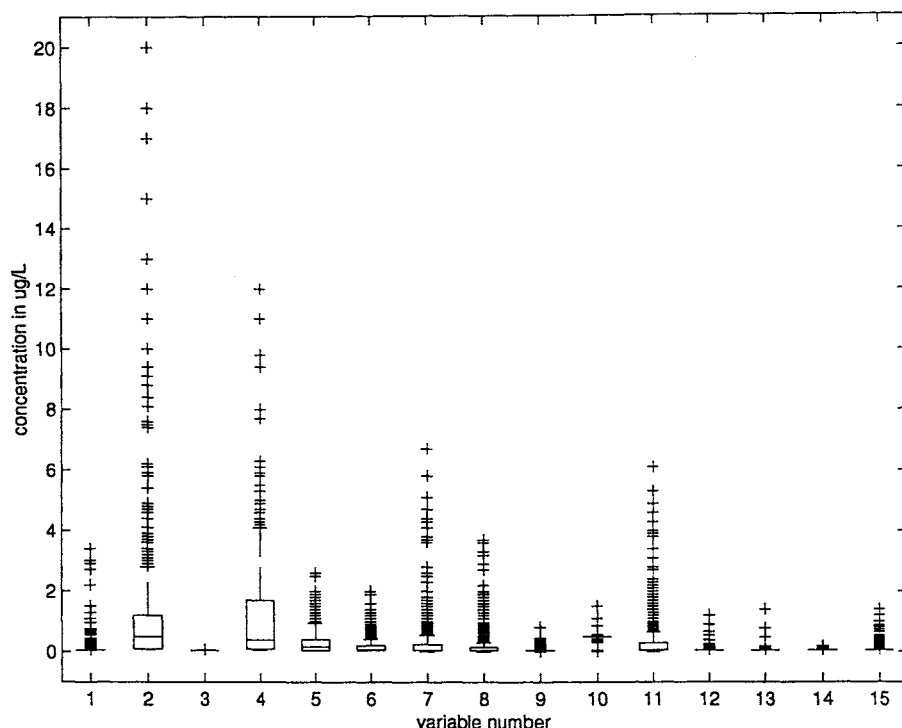
Fig. 16.11. Box plot of measured concentrations. For each variable, the box has lines at the lower quartile, the median and upper quartile values. The whiskers are the lines extending from each end of the box to show the extent of the rest of the data (US Midwest reservoirs study [72]).

total). For cyanazine (variable 7) and metolachlor (variable 11), only one missing value was present. For the other variables no missing values were present. It turns out that for variables 8, 9 and 10, the same entry values were missing; this means that the analyte concentrations corresponding to these entries were systematically not reported for these three variables. Looking at the original data, these values usually corresponded to the measurements performed in early winter (January) or summer (June or July), but was not the same for all the reservoirs. Missing values were handled using PCA. First, missing values were set to zero and a PCA model was calculated for the whole data set and the data reproduced using this model. The missing values were then replaced with those values, which were most consistent with the PCA model. A new model was then recalculated, and the process was repeated until the estimates of the missing data converged. To evaluate the missing values a PCA model of five components was used explaining practically all the experimental data variance (99.9%).

As all the analyte concentrations were measured in the same scale units and apportionment of source contributions was intended, initial data analysis was performed without any scaling nor mean-centering. Comparison of the results obtained in this way with those obtained using data scaling was also performed. Since a large part of the data values were only positive and near the limit of detection of the measurements (LOD values), log data transformation was also examined and the results compared.

Values below the limit of detection were assumed to be positive and equal to the LOD. Approximately half of the entries in the original data were below the limit of detection (approximately 52%). In the calculations, these values were set equal to the LOD values for that variable (analyte concentration) or to its half. Both strategies were tested and gave the same results. Values below LOD were not equally distributed among variables. For variables 1, 3–9 and 11–15, the LOD was equal to 0.05 μg/l. For variable 2 (alaESA), LOD was 0.1 μg/l and for variable 10 (deethylcyanazineamide) LOD was 0.5 μg/l. Variable 10 is especially problematic because it has a large number of missing values and a large number of values below LOD (96%). Only 26 from 608 values were higher than the LOD of this variable. Variable 3 is also very problematic since only two values were above its LOD. Other variables with few values above LOD were variables 12, 13, 14 and 15. It is obvious from this preliminary study that the more reliable variables were variables 1, 2, 4, 5, 6, 7, 8, 9 and 11. The whole data analysis was performed either using all variables or using only those nine more reliable and informative variables.

The correlation between sets of two variables was investigated to see the interrelationships between different analyte concentrations in the different analyzed samples (Table 16.2). This was accomplished simply by calculating the correlation coefficients between all the values corresponding to two-selected variables and this was repeated for all possible sets of two variable combinations. Very high correlations (>0.9) existed between the concentrations of atrazine and the concentration of its derivatives, DEA and DIA. High correlations (>0.8) were also observed between the concentrations of metolachlor, alachlor and atrazine and its metabolites (DEA and DIA), and between cianazine and its derivatives. Alachlor metabolite concentration, alaESA, did not show a high correlation (<0.8) with any of the other analyte concentrations, not even for alachlor (0.69), which indicates that the behavior of this analyte is rather independent from the others.

For the whole data set, the first PCA component already explained 84.59% of the total variance (Table 16.3), the second component 9.82% and the third 2.40%.With two and three components 91.41% and 94.4% of the total variance was already explained. The fourth component explained a little more than one percent (1.5%) and the fifth component explained below 1% of the total variance. Therefore, most of the data variance was explained by the first three components, showing that the information provided by the

TABLE 16.2

CORRELATION BETWEEN THE NINE MORE IMPORTANT VARIABLES (US MIDWEST STUDY [72])

	Ala	AlaESA	Atraz	DEA	DIA	Cian	Cianam	Deethyl	Metol
Ala	1.00								
AlaESA	0.69	1.00							
Atraz	0.70	0.68	1.00						
DEA	0.61	0.73	0.92	1.00					
DIA	0.63	0.73	0.86	0.92	1.00				
Cian	0.55	0.57	0.69	0.67	0.78	1.00			
Cianam	0.52	0.62	0.69	0.71	0.80	0.85	1.00		
Deethyl	0.45	0.47	0.60	0.5	0.66	0.84	0.83	1.00	
Metol	0.81	0.73	0.84	0.80	0.81	0.65	0.64	0.51	1.00

TABLE 16.3

PERCENTAGE OF CUMULATIVE VARIANCE EXPLAINED BY PCA RESULTS (US MIDWEST STUDY [72])

Matrix	PC1	PC2	PC3
D 15 variables	84.59	94.41 (9.82)[a]	96.80 (2.40)
Dr[b] 9 variables	85.81	95.75 (9.94)	98.16 (2.41)
D autoescaled	50.97	59.03 (8.06)	66.16 (7.13)
Dr autoescaled	73.62	84.57 (10.95)	89.84 (5.27)
D scaled	99.56	99.80 (0.24)	99.84 (0.04)
Dr scaled	79.66	87.74 (8.09)	91.68 (3.94)
D log transformed	91.42	97.02 (5.60)	97.92 (0.90)
Dr log transformed	89.47	95.65 (6.18)	97.16 (1.51)

[a] The non-cumulative variance is given in parentheses.
[b] Reduced data set (only 9 of the 15 variables are examined).

15 variables was correlated. The first PC had high loadings (Fig. 16.12) on variable 2 (alaESA) and variable 4 (atrazine). The second PC also had high loadings by variables 2 and 4 but in an inverse way. While in the first PC, variables 2 and 4 were positively correlated (both increasing or decreasing simultaneously), in the second PC, the same two variables were negatively or inversely correlated (Fig. 16.12), i.e. when one increased the other decreased or inversely. The third PC was highly loaded by variable 7 (cianazine). Metolachlor loaded both PC1 and PC2. Similar observations could be deduced from loading biplots, which showed that variables 2, 4, 7 and 11 were the more determinant in the data description and in the data variance interpretation. Most of the samples have low score values. However, some samples had high values for the first score (i.e. sample 151, from Salmonie Lake, Indiana, measured in July) or high values for the second score (i.e. sample 79, corresponding to sample taken in July in Cataract Lake, also in Indiana). This meant that these two samples had high inputs in variables 2 (alaESA) and 4 (atrazine), although probably not coming from the same origin. Scores for the third PC (high cyanazine) showed a similar trend for most of the samples except for some samples like sample 282 (sample taken in late June in Lac Qui Parle Reservoir, Minnesota) with a much higher value for this third PC score. PCA was repeated considering only the 9 more significant variables (variables 1, 2, 4, 5, 6, 7, 8, 9 and 11) after elimination of the six less significant and less reliable variables (3, 10, 12, 13, 14 and 15). PCA results obtained with this new reduced data set were similar to results given above, now with a total explained variance of 85.81%, 95.75% and 98.16%, respectively, for the first three principal components. Whereas the elimination of these six variables had a small effect in the PCA results, when no transformation is applied to the data, for the scaled and autoscaled transformed data matrices, the reduction of the number of variables had a significant effect. This was due to the fact that the scaling of the variables with most of their values close to the limit of detection gave very unreliable results. For instance, for the whole autoscaled an scaled data, variables less reliable like variable 3 (ametryn), 10 ((deethylcyanazineamide), and 12–15, gave high loadings for the firsts PCs. The reason why this happened was because when these variables were scaled (divided by their very small standard deviation), their
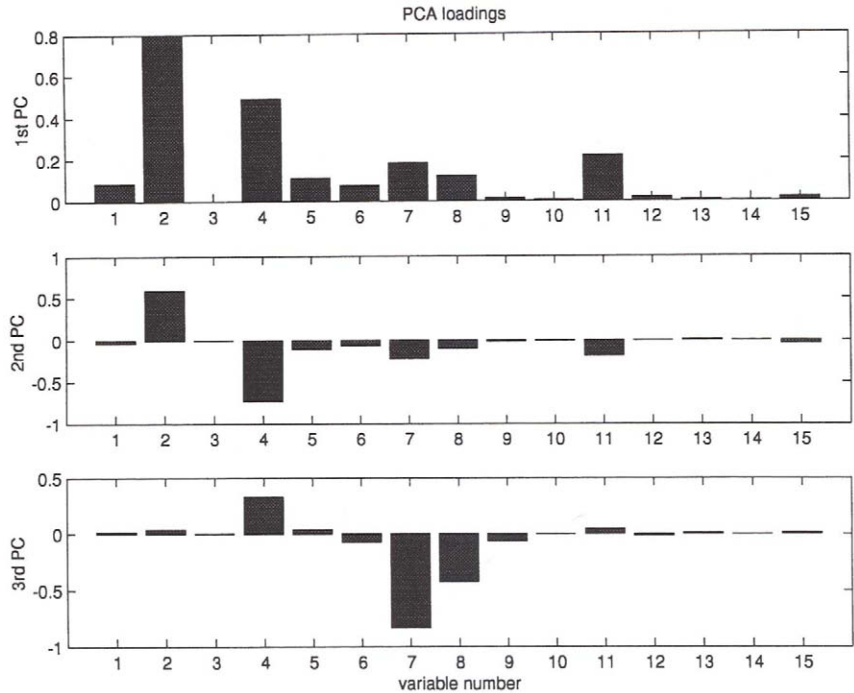
Fig. 16.12. PCA loadings of the whole column-wise augmented data matrix (US Midwest reservoirs study [72]).

values become very large. For the reduced number of variables, data set, scaling and autoscaling pretreatment gave similar loading values. In this case all the variables contributed similarly to the first PC with high loadings, variables 8, 1 and 9 were more important in the second PC and variables 1, 4 and 6 in the third PC. Data interpretation was more difficult when experimental data were scaled than when they were not and the information and variance coming from high input concentration variables was mostly lost. Also, in Table 16.3, the results of PCA analysis for log transformed data are given both for all and for the reduced variables data sets. For log transformed data the effect of variable reduction in terms of explained variance was much lower than for scaled and autoscaled data. No advantages were noted for log transformed data. In this case the first PC had again high loadings from most of the variables, the second PC mostly from variables 2, 3 and 8 and the third PC from variables 1, 2, 3, 6, 7 and 9. As the goal of the analysis was to distinguish the possible different sources of data variance, neither of the proposed data transformations gave better results that the non-transformed reduced data matrix (see a summary of PCA results in Table 16.3).

MCR–ALS results were interpreted in terms of resolution of composition profiles of the three possible sources of data variation and in terms of contributions profiles or distributions (temporal and geographical) of the detected variation sources. These profiles were not forced to be orthonormal and explaining maximum variance as in PCA, but only to be non-negative and explaining all three together maximum variance (non-negative least

squares solution [57,58]). Whereas PCA profiles were mathematical solutions, which probably do not agree with real sources of variation, ALS profiles attempted to recover the real (physically meaningful) source profiles. Fig. 16.13 gives the three more important ALS resolved source composition profiles when non-negative constraints were used in the ALS decomposition of the whole augmented data matrix. Initial estimates for the ALS optimization were obtained from the detection of the purest samples [40,41]. The three selected samples were the sample number 104 (high concentration for alaESA), the sample number 321 (high concentration for atrazine) and the sample number 282 (high concentration for cyanazine). Similar results were obtained if the ALS optimization was started with the purest variables. In the later case, the selected variables were 1, 7 and 15, respectively. The explained variance achieved using three components and non-negative constraints was also 96.8%, i.e. similar to that obtained when PCA was applied for the same number of components. Source composition profiles given in Fig. 16.13 show that the first resolved profile accounted mostly for alaESA and some metholachlor, second resolved profile accounted for atrazine and also for some metholachlor and deethylcyanazineamide and the third resolved composition profile accounted mostly for cyanazine, atrazine and some metolachlor.

Source contributions were plotted in two different ways (Figs. 16.14 and 16.15) depending on whether the interest was to look at the time distributions or to the geographical-reservoir distributions. For a large number of cases the time distributions were low close to
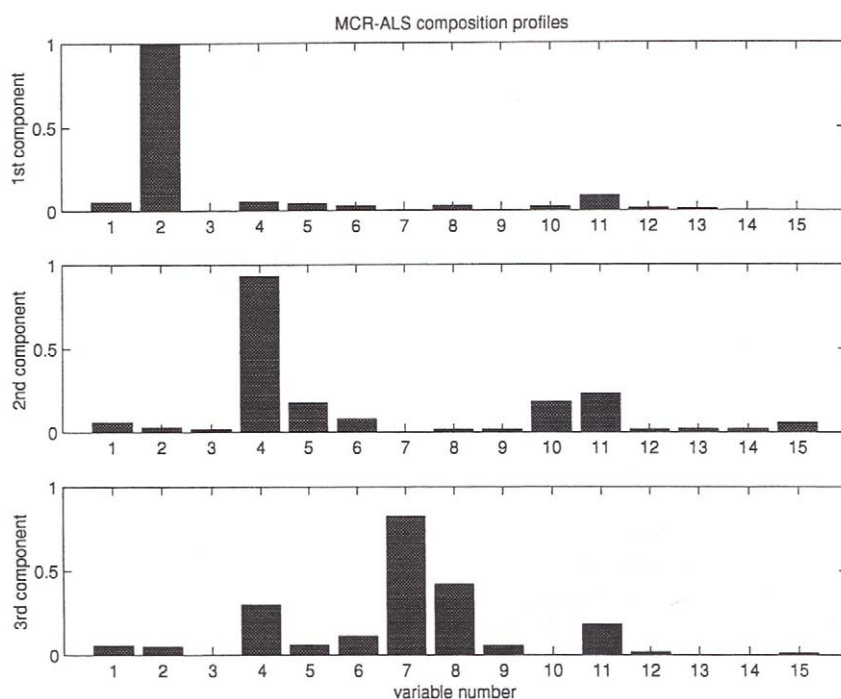
Fig. 16.13. MCR–ALS resolved source composition profiles (matrix $\mathbf{S}^T$) obtained for the data of Fig. 16.11 (US Midwest reservoirs study [72]).
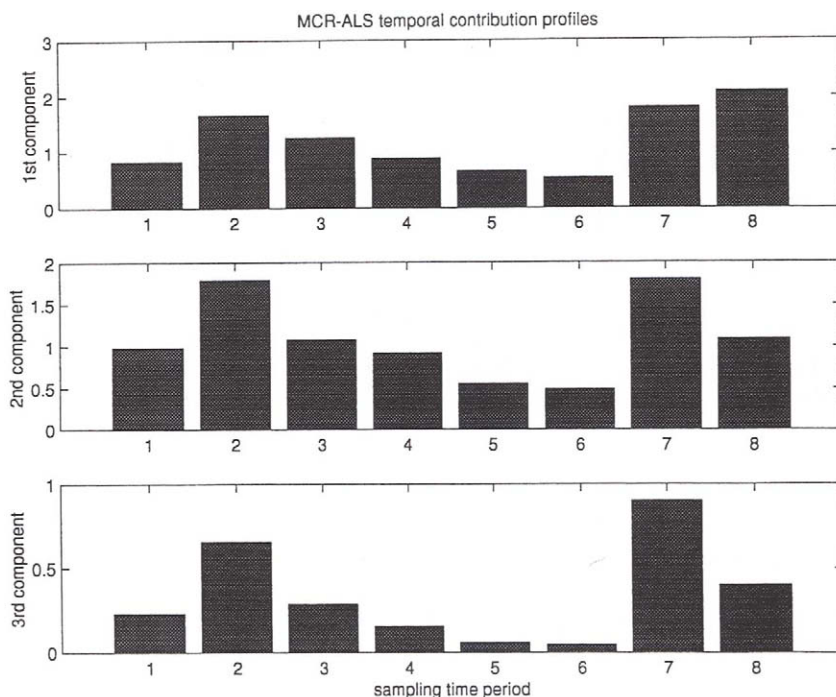
Fig. 16.14. MCR–ALS resolved time contributions resolved for the three main sources of data variation and averaged for the 76 reservoir sampling sites and obtained for the data of Fig. 16.11 (US Midwest reservoirs study [72]).

what seemed to be the background contribution level. However, for a significant number of time profiles, there was a clear and repetitive seasonal pattern with the highest values in summer 1992 and in summer 1993. Similar patterns were also shown for the time distribution profiles of the second (with high atrazine content) and for the third resolved time profile (with high cyanazine content). From Fig. 16.14 it was confirmed that the peak concentrations of the herbicides and metabolites were obtained in the summer season. Fig. 16.15 gives the average distribution per reservoir of the three main environmental sources of herbicides and metabolites during the investigated time period. Reservoirs in the Indiana region (sites 15, 17 and 19) gave high values for the three environmental sources. Some reservoirs in the Ohio region (sites 61, 63, 65) gave relatively high values for the first resolved environmental source but not so high for the other resolved sources. Reservoir 41 in Missouri gave a high value for the second resolved source and reservoir 51 in Nebraska gave a high value for the third resolved source. Looking in detail the plots given in Figs. 16.14 and 16.15, it is easy to have a fast, easy and comprehensive comparison of the geographical distribution of the environmental sources of herbicides and their metabolites in the whole studied region.

When the trilinearity constraint was applied during the ALS optimization, Eq. (19) the percentage of explained variance for three components dropped down to 86.92%, 10% below the value obtained when trilinearity constraint was not applied. However, the
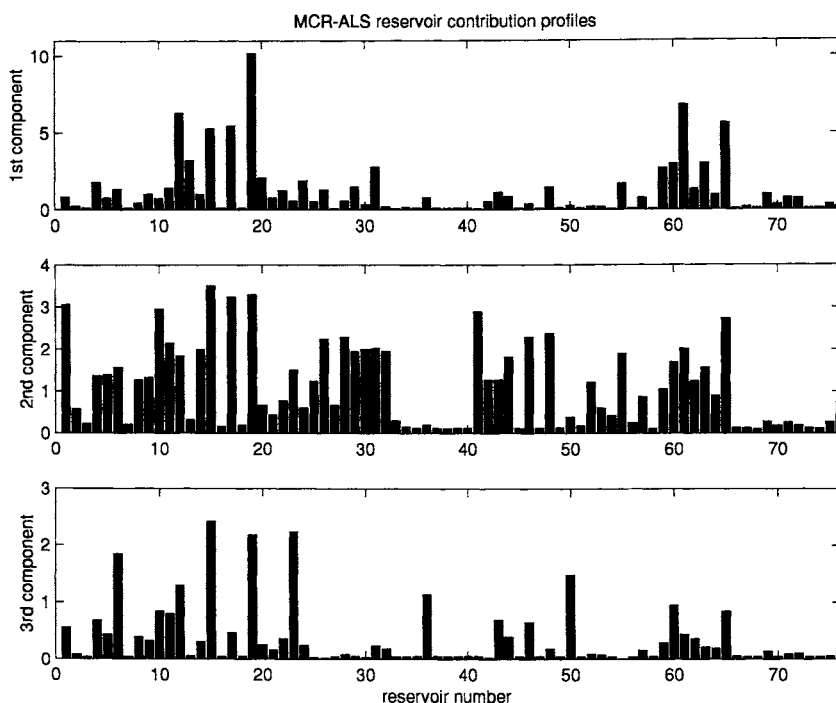
Fig. 16.15. MCR–ALS resolved reservoir contributions resolved for the three main sources of data variation and averaged for the 8 sampling time periods and obtained for the data of Fig. 16.11 (US Midwest reservoirs study [72]).

resolved composition profiles were very similar to those obtained when no trilinearity constraint was applied (Fig. 16.13). The same can be said for the contribution profiles, both temporal and reservoir distributions. This seems to indicate that although the data were not strictly trilinear (this is the reason why the percentage of explained variance drops considerably) the shape of the time distributions of pesticide and herbicide concentrations was very similar for all reservoirs. Assuming trilinearity or not, produced essentially the same resolved composition and contribution profiles.

Finally, when the MCR–ALS analysis was applied to the reduced number of variables data set, the same results were obtained, also with a considerable difference in the percentage of explained variance when trilinearity constrains were applied or not. When data pretreatment like log transformation was applied, interpretation of resolved profiles did not provide any new insight in the interpretation of the observed data variance.

16.3.3 Resolution of pesticide degradation products by means of spectrometric methods [75]

Methyl (1-butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) is widely used as a systemic fungicide for crop diseases. Due to its extensive use and carcinogenic activity the determination of benomyl and its main degradation compound 2-benzimidazolecarbamate (carbendazim) has been the object of several studies in environmental water, soil extracts

and crops [76]. Because of its instability in organic solvents and its low solubility in water several approaches have been made to understand the degradation path of benomyl in different media. Most methods reported in the literature determine benomyl by chromato-graphic methods after its conversion into a stable degradation compound. This approach is based on the conversion of benomyl to carbendazim, although it does not allow one to distinguish carbendazim resulting from the degradation of benomyl during the sample analysis from carbendazim present in the samples as a natural product of degradation [77]. The conversion of benomyl to other degradation products such as 3-butyl-2,4-dioxo-s-triazino(1,2-a(benzimidazole (STB), to 1-(2-benzimidazolyl)-3-n-butylurea (BBU) and to 2-aminobenzimidazole (2-AB) has also been reported. Most of the degradation reactions of pesticides in water follow an oxidation process, producing more polar compounds than the parent one. These compounds are not sufficiently volatile to be analyzed by gas chromatography. The use of LC–DAD can overcome this problem as it allows the analysis of more polar compounds. In addition, the combination of solid-phase extraction (SPE) with LC–DAD allows the analysis of pesticides in a low level concentration similar to that found in environmental waters. Kinetic degradation of benomyl can be also monitored by UV spectrophotometry. In this case the spectra recorded at preselected time intervals evolve as the degradation reaction takes place, with noticeable changes in the absorption bands. However, the information provided by UV absorption spectrophotometry is unre-solved and no selective wavelengths for benomyl or its degradation products are expected. To solve this problem, the mathematical resolution of the mixture of benomyl and its degradation products by means of factor analysis and multivariate curve resolution (MCR) methods is proposed and shown in this work. The aims were the study of the degradation of benomyl and carbendazim at different pH values by UV spectrophotometry and chemo-metric methods.

Spectrophotometric measurements were performed in a lambda-19 UV-VIS spectro-photometer from Perkin Elmer (Norwalk, CT, USA). The spectra were registered from 200 to 320 nm, with a resolution of 1 nm, every 15 min during 12 h. Temperature was set with a control temperature device at 25°C. The samples were prepared by dissolving carbendazim in aqueous solution adjusted to pH, 3, 4.5, 6, 7.5 and 9. In order to ensure complete solubilization of carbendazim, 10 ml of methanol were added to a total volume of 100 ml. The first spectrum of the sample was recorded 5 min later from the beginning of the dissolution process to achieve the same degree of degradation in all the samples. A blank of the solvent was registered at the beginning of each set of spectra. Sample concentration was 1.0×10^{-5} M. The conversion of benomyl to its related degradation compounds was studied spectrophotometrically during 12 h at five fixed pH values: 3, 4.5, 6, 7.5 and 9. In Fig. 16.16 the set of spectra recorded at pH 7.5 is shown.

The number of absorbing species formed by degradation of benomyl was first estimated. The more reliable estimation of the total number of species was obtained by the simulta-neous analysis of the degradation process at different pH values. In this way, the species, which were at low concentrations at a particular pH, could be detected at larger concen-trations at other pH values. From the percentage of lack of fit using PCA and MCR–ALS (Table 16.4), it was concluded that the total number of more probable species at the different pH values was four. However, not all the species were present (at high concen-trations) at all the investigated pH values. Resolved spectra and resolved concentration profiles using the ALS procedure are shown in Figs. 16.17 and 16.18. Species 1 was
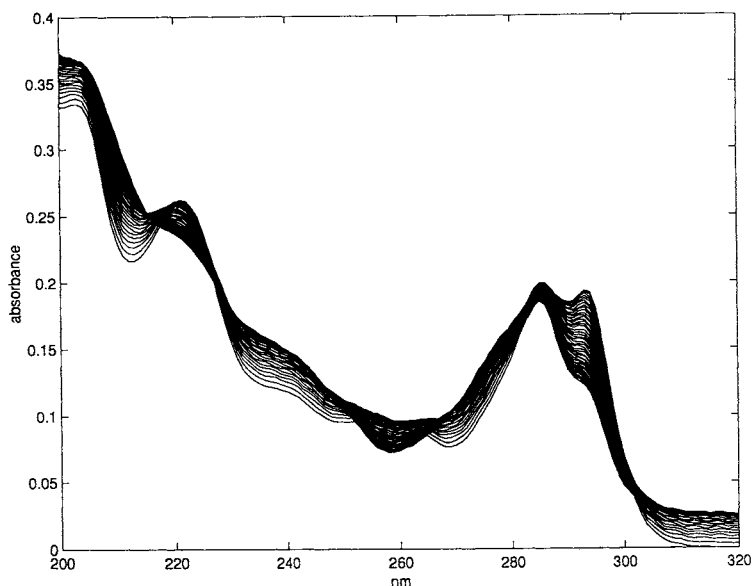
Fig. 16.16. Study of the degradation of benomyl in water at pH 7.5 using UV spectrometry (see [75] for more details).

TABLE 16.4

PERCENTAGE OF LACK OF FIT[a] OBTAINED BY PCA AND MCR–ALS CONSIDERING DIFFERENT NUMBER OF COMPONENTS AND FOR DATA MATRICES AT DIFFERENT pH VALUES[b] (PESTICIDE DEGRADATION STUDY [75])

No. of PCs	PCA % *lof*					ALS % *lof*			
	1	2	3	4	5	2	3	4	5
P3	9.59	0.51	0.35			0.55			
P4	5.90	0.86	0.18			0.86	0.88		
P6	6.02	0.64	0.16			0.64	0.30		
P7	5.29	1.05	0.21			1.05	0.36		
P9	4.13	0.26	0.19			0.26			
P34	9.37	5.64	0.89				0.94	0.52	0.32
P346	8.13	5.97	1.79				2.2	0.43	0.22
P3467									
P34679	17.18	4.08	6.50	1.58	0.77			2.18	

$$^a lof = \sqrt{\frac{\sum (d_{ij} - d_{ij}^c)^2}{\sum d_{ij}^2}} \times 100$$

where d_{ij} are the experimental values and d_{ij}^c are the corresponding calculated values using PCA or MCR–ALS.

[b] PCA and MCR–ALS results obtained for the single data matrices p3, p4, p6, p7 and p9, corresponding to pH values 3, 4.5, 6, 7.5 and 9, respectively, and for the augmented matrices p34 (pH 3 and 4.5), p346 (pH 3, 4.5 and 6), p3467 (pH 3, 4.5, 6 and 7.5) and p34679 (pH 3, 4.5, 6, 7.5 and 9).

identified as benomyl since its resolved spectrum was practically identical to that of pure benomyl. Species 3 was also identified as carbendazim since its spectrum was also identical to that previously known for the same compound. Species 2 was identified as a protonated form of carbendazim (it has common bands with carbendazim and it only appears at low pH). Species 4 was attributed to a metabolite of benomyl that appears at basic pH, probably 3-butyl-2,4-(1,2a)-s-triazinobenzimida-zole (STB).The distribution of the species at pH 4.5, 6 and 7.5 showed common species, benomyl (species 1), carbenda-zim (species 3) and the new compound (species 4) whose concentration increased with the increase of pH. A very fast conversion of species 1 (benomyl) to species 4 (STB) at pH 9 was observed, whereas this conversion was negligible at pH 3. The appearance of the protonated carbendazim species (species 2 in Fig. 16.17) as the main degradation species at pH 3 was in agreement with the reported pK_a for carbendazim in the literature as 4.2. This species was not present at pH 7.5 and is not shown in Fig. 16.18. From the resolved concentration profiles, the relative amounts of each species at different times could be easily estimated. In particular, the half-life of benomyl, estimated as the time when its concentration is the half, was estimated at different pH values: 25 min at pH 3, 18 min at pH 4.5, 14 min at pH 6, 12 min at pH 7.5, and less than 1 min at pH 9. As expected the degradation of benomyl was much faster at basic pH values.

16.3.4 Resolution of chromatographic unresolved mixtures of pesticides [78]

Multivariate curve resolution (MCR) has been also used for the resolution and quantitation of strongly coeluted compounds in liquid chromatography with diode array (LC–DAD [62,78–80]) or mass spectrometry (LC–MS [81]) detection. In the present work, the MCR method was applied to handle real liquid chromatographic data at low concentrations (ng/l) of herbicides using preconcentration techniques. The goal of the study was the resolution and quantitation of metholachlor and alachlor in mixtures at low concentrations using in-line solid-phase preconcentration techniques. These two herbicides have very similar spectra and coelute strongly, especially at low concentrations and when preliminary on-line solid-phase preconcentration techniques [82] are used. Interferents at very low concentrations and solvent effects become more significant in preconcentration techniques than for direct injection analysis of more concentrated samples.

Experimental details are given in [83]. Two chromatographic columns were used: Waters (Chromatographic runs and data matrices W1–W9) and Merck (Chromatographic runs and data matrices M1–M3, M8 and M9); see Table 16.5. As chromatographic conditions were not completely reproducible and coelution produced changes in shapes and retention times of the chromatographic profiles, the selection of the particular elution time ranges included in each data matrix was done empirically. Elution times having the desired cluster of peaks (identified by the their spectra) were initially selected. Each chromatographic run provided a data array of numbers which were ordered in a data table or matrix with a number of rows equals to the number of selected elution times and a number of columns equal to the number of wavelengths. Whereas for all the runs the same spectral range was selected (190–250 nm, 51 wavelengths, resolution of 1.2 nm), a different number of elution times was selected for each data matrix depending on the elution of the components of interest. Thus, all data matrices had the same number of columns but
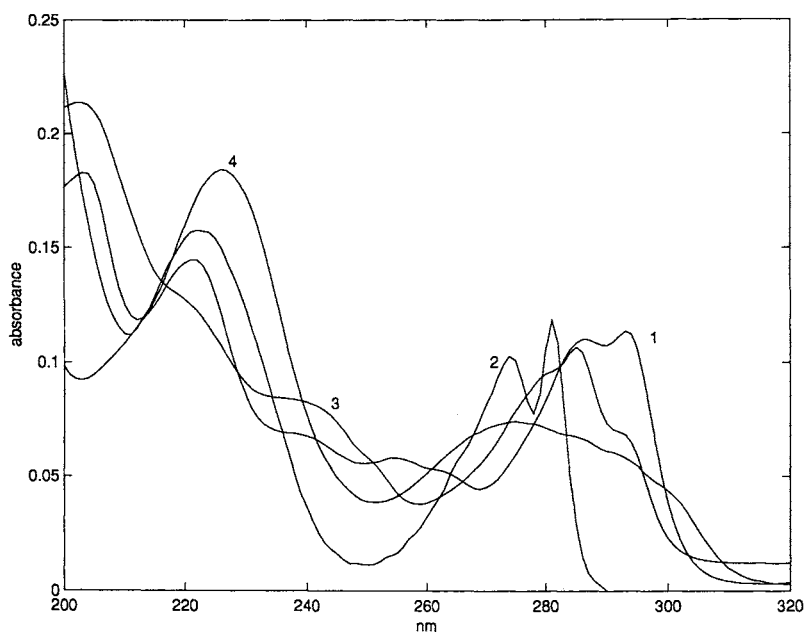
Fig. 16.17. MCR–ALS resolved species spectra of benomyl and its degradation products. Plotted species are: 1, benomyl; 2, protonated carbendazim; 3, carbendazim; 4, STB (reproduced from [75]).
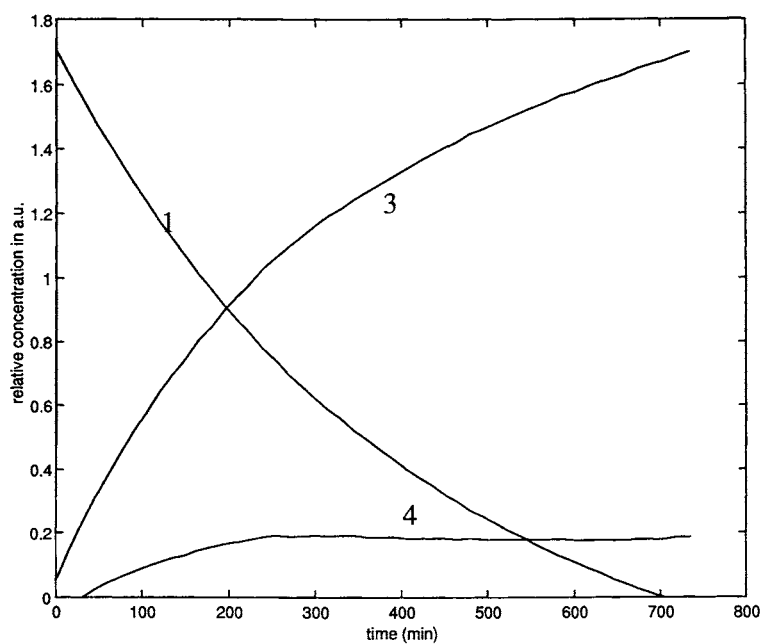
Fig. 16.18. MCR–ALS resolved concentration profiles of benomyl and its degradation products at pH 7.5 (see Fig. 16.16). Plotted species are: 1, benomyl; 3, carbendazim; 4, STB (reproduced from [75]).

TABLE 16.5

EXPERIMENTAL DETAILS OF DIFFERENT DATA MATRICES (LC–DAD STUDY)

Chromatography column[a]	Data matrix[b]	Composition[c] (ng)		Matrix[d]	Method[e]
		ala	Met		
Merck	M1(81,51)	404	–	mq	1
Merck	M2(51,51)	–	400	mq	1
Merck	M3(101,51)	404	400	mq	1
Merck	M8(66,51)	150	151	Delta Ebre	2
Merck	M9(66,51)	–	–	Delta Ebre	2
Waters	W1(61,51)	404	–	mq	1
Waters	W2(41,51)	–	400	mq	1
Waters	W3(41,51)	404	400	mq	1
Waters	W4(41,51)	–	–	mq	2
Waters	W5(41,51)	150	–	mq	2
Waters	W6(41,51)	–	151	mq	2
Waters	W7(41,51)	150	151	mq	2
Waters	W8(34,51)	150	151	Delta Ebre	2
Waters	W9(34,51)	–	–	Delta Ebre	2

[a] Chromatographic columns used in the analysis.

[b] Data matrices used in the analysis. In parentheses for each data matrix, the number of rows (elution times at every 1 s) and the number of columns (51 wavelengths, between 190 and 240 nm, every 1.2 nm) are given.

[c] Composition of the analyzed samples given as total amount of the analytes input to the column. Ala gives the amount of alachlor and met gives the amount of metholachlor.

[d] Samples W1,W2,W3,W4,W5,W6, M1, M2 and M3 are prepared with pure water mQ. Samples W8, W9, M8 and M9 are real water samples taken from Delta river and spiked with the given amounts of pesticides.

[e] Sampling method: 1, direct injection of the a standard sample to the column without preconcentration; 2, on-line solid-phase PROSPECT preconcentration previous to injection to column [78,82,83].

they could have different number of rows. Time resolution in the data analysis is of 1 s (for every spectrum).

Background absorption was detected for all data matrices, especially for those data matrices obtained using preconcentration techniques and for chromatograms at lower wavelengths. Most of the background contribution was caused by the absorption of the solvent whose composition and relative concentration changed along the chromatographic elution. To subtract the initial background absorption caused by the solvent, the spectrum at the first initial elution time was subtracted from subsequent spectra at all other elution times. In this way all the chromatograms started at zero but owing to gradient elution, a small background contribution increased with time. In Fig. 16.19 a plot of the chromatograms obtained at different wavelengths for the data matrix W8 (Ebre Delta sample, Table 16.5). A maximum number of five components (Table 16.6) were detected for data matrices W7 and W8. Of these five compounds, the first was the elution of metholachlor. Second was the elution of an unknown contribution only present when preconcentration techniques were used. Third was the elution of alachlor, and fourth and fifth minor contributions were related to the solvent, to the gradient elution and/or to the subtraction procedure. Matrices W5 and W6 (Table 16.5) had a maximum of four components because
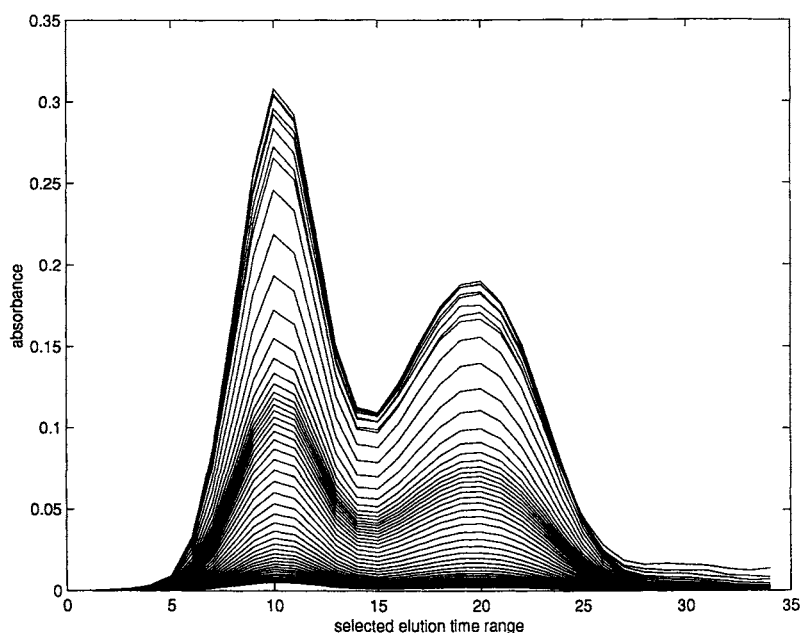
Fig. 16.19. LC–DAD analysis of an Ebre Delta river water sample (spiked with alachlor and metolachlor) corresponding to data matrix W8 (see Table 16.6, reproduced from [78]).

one of the two analytes (alachlor or metholachlor) was not present. Blank data matrices W4 and W9 had a maximum of three components (unknown interferents and/or a solvent contributions, Table 16.6). Matrix W3 had three components, the two analytes and a very low solvent contribution. Matrices W1 and W2 had two components respectively, one of the two analytes and a very low solvent contribution. Similar results with a better resolution of analytes, interferents and solvent were obtained using a Merck column (data matrices M1, M2, M3, M8, M9 of Table 16.5). Matrices M8 and M9 gave four components (only one solvent contribution is detected for the Merck column, Table 16.6). Matrices M1 and M2 had only a major contribution and a very low solvent contribution; M3 had three components, the two analytes and the solvent. Small PCA residuals and unexplained variance were found always except for blank matrices W4, W9 and M9, where the signal to noise ratio in those blank matrices is much lower.

Normalized pure spectra of the two analytes, metholachlor and alachlor, were estimated from the individual MCR–ALS analysis of matrices W1, W2, M1 and M2 (without preconcentration). The spectra of these two components were found to be extremely similar (Fig. 16.20) with a correlation coefficient equal to 0.9992. They were used as input values in the analysis of the other data matrices and confirmed in the analysis of matrices W5 and W6 (similar to W1 and W2 with only one of the two analytes but using the preconcentration method). The analysis of the blank data matrices W4, W9 and M9 provided the initial estimations of the unknown spectra contributions. Once these components were identified, multivariate curve resolution of coeluted components in the mixture matrices W3, W5 and W8 was attempted. Resolution for both alachlor and metholachlor was achieved in the individual analysis of data matrices W7, W8, M3 and M8. For matrix

TABLE 16.6

INDIVIDUAL ANALYSIS OF CHROMATOGRAPHIC RUNS (LC–DAD STUDY) USING PCA AND MCR–ALS

Matrix[a]	No. of comp.[b]	PCA lof^c (%)	ALS lof^c (%)	Species[d]
W2	2	0.19	0.44	1,5
W3	3	0.16	–[e]	1,3,5
W4	3	1.94	3.97	2,4,5
W5	4	0.25	0.65	2,3,4,5
W6	4	0.32	0.52	1,2,4,5
W7	5	0.17	1.14	1,2,3,4,5
W8	5	0.24	2.18	1,2,3,4,5
W9	3	2.55	2.71	2,4,5
M1	2	0.31	0.59	3,5
M2	2	0.19	0.90	1,5
M3	3	0.25	0.75	1,3,5
M8	4	0.43	4.79	1,2,3,5
M9	4	9.99	1.50	2,5

[a] Experimental data matrix analyzed (see Table 16.5 for notation).

[b] Number of components found in the principal component (PCA) ternating least squares analysis (ALS).

[c]

$$lof = \sqrt{\frac{\sum (d_{ij} - d_{ij}^c)^2}{\sum d_{ij}^2}} \times 100$$

where d_{ij} are the experimental values and d_{ij}^c are the corresponding calculated values using PCA or MCR–ALS. Constraints used in the ALS optimization are non-negativity for both concentration and spectra profiles, and unimodality except for matrices W4, W9 and M9.

[d] Identification of the species following their elution order: 1st is metholachlor; 2nd is an interferent found in the on-line preconcentration experiments; 3rd is alachlor; 4th is another interferent found in preconcentration experiments using Waters column; and 5 is a solvent contribution appearing at the end of the elution due to the gradient elution and to the subtraction procedure used in the data pretreatment.

[e] Resolution and optimization of the elution profiles of both components, metolachlor and alachlor, fails.

W3, however, resolution failed to converge to a reasonable solution, probably because of the higher overlap of these two compounds in this data matrix. The individual analysis of the different data matrices using the two columns showed that: (1) the analysis was independent of the column used; (2) solvent contributions were mostly due to gradient elution; (3) unknown coeluted contributions appeared when on-line solid phase precon-centration methods were used. In Table 16.6, a summary of the results obtained in the individual analysis of the different chromatographic runs is given.

In the simultaneous analysis of several data matrices (Table 16.7), the resolution power was improved respect the individual analysis of the different data matrices and the relative quantitation of the different analytes was possible. The simultaneous analysis of chroma-tographic runs W1,W2 and W3 without preconcentration (augmented matrix [W1;W2;W3]) allowed the resolution of the two analytes, alachlor and metholachlor, even for matrix W3, which could not be resolved in the individual analysis. A small contribution of the solvent was also confirmed. The resolution between the two profiles of these compounds in matrix W3 was very low (around 0.2). Even for this unfavorable
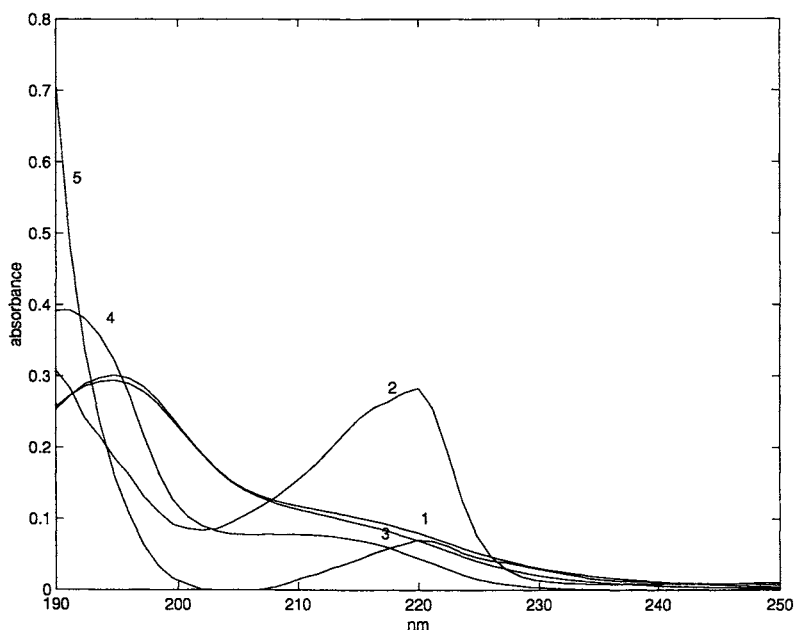
Fig. 16.20. MCR–ALS normalized spectra for the resolved species: metholachlor (1), unknown interferent found in preconcentration experiments (2), alachlor (3), unknown interferent or mobile phase contribution found in the preconcentration experiments with the Waters column (4) and mobile phase contribution due to gradient and first spectrum subtraction (5) (reproduced from [78]).

case of strongly very similar coeluted compounds (see correlations in Table 16.8) a good resolution and quantitative estimation was achieved for the two analytes. The same can be said for M3 matrix when analyzed together with matrices M1 and M2 (augmented matrix [W3;W1;W2]) although now (Merck column) the resolution was higher ($R_s = 0.57$). When one of the single analyte matrices was omitted from the analysis (W2 or W1 in the analysis of W3 or M1 or M2 in the analysis of M3) the results were still good for the quantitation of the common component (see Table 16.8). This proves the power of the proposed method even in the very unfavorable case of quantitation in the presence of a very similar coeluted interferent compound. Simultaneous analysis of chromatographic runs of preconcentrated water samples spiked with the same amount of very low concentrations of the analytes alachlor and metholachlor, augmented [W7;W4;W5;W6] matrix, allowed the resolution of the coeluted components and the quantitation of alachlor and metolachlor in their mixtures. Samples only having one of the two analytes were takes as standards. Percentage errors in the quantitative estimations are given Table 16.7. When one of the single analyte matrices, W5 or W6, is omitted from the augmented matrix, quantitative estimations are still good (see results for the analysis of the augmented matrices [W7;W4;W5]), even when blank information is also omitted (augmented matrices [W7;W5] or [W7;W6]). Simultaneous analysis of preconcentrated Delta river water samples (W8) spiked or not (W9) with the two analytes together with other water samples (augmented matrix [W8;W9;W7;W4;W5;W6;W3;W2;W1]) gave similar results to those obtained previously. These results were also illustrative of the comparison

TABLE 16.7

SIMULTANEOUS ANALYSIS OF DIFFERENT CHROMATOGRAPHIC RUNS BY MCR–ALS (LC–DAD STUDY)

Column	Matrix	ALS % lof[a]	Metholachlor[b] (%)	Alachlor[b] (%)
I Waters	[W3; W1; W2]	1.57	381 (5)	396 (2)
I Waters	[W3; W1]	1.60		392 (3)
I Waters	[W3; W2]	0.86	396 (1)	
II Waters	[W7; W4; W5; W6]	5.09	159 (5)	151 (1)
II Waters	[W7; W4; W5]	4.59		144 (4)
II Waters	[W7; W5]	2.25		136 (10)
II Waters	[W7; W6]	8.41	156 (3)	
III Waters	[W8; W9; W7; W4; W5; W6; W3; W2; W1]	3.26	154 (2)	158 (5)
			146 (3)	141 (6)
			420 (5)	368 (9)
			410 (2)	383 (2)
III Waters	[W8; W1; W2]	2.31	161 (7)	152 (2)
	[W8; W1]	3.00		144 (4)
	[W8; W2]	1.44	172 (13)	
III Waters	[W8; W3]	1.85	160 (6)	154 (2)
IV Merck	[M3; M1; M2]	1.54	425 (6)	400 (1)
IV Merck	[M3; M1]	1.14		408 (1)
IV Merck	[M3; M2]	1.23	430 (7)	
IV Merck	[M8; M9; M3; M1; M2]	3.09	140 (7)	149 (1)
			437 (9)	387 (4)
IV Merck	[M8; M1]	4.43		135 (10)
IV Merck	[M8; M2]	2.51	150 (1)	
V Waters–Merck	[W3; W1; W2]	1.70	416 (4)	385 (5)
	[M3; M1; M2]		428 (7)	404 (1)
			424 (6)	427 (6)
VI Waters–Merck	[W8; M2]	1.90	166 (10)	

a

$$lof = \sqrt{\frac{\sum(d_{ij} - d_{ij}^c)^2}{\sum d_{ij}^2}} \times 100$$

where d_{ij} are the experimental values and d_{ij}^c are the corresponding calculated values using PCA or MCR–ALS.
   [b] Quantitation values obtained by comparison of resolved profiles areas. Errors in the quantification in parentheses.

between preconcentrated water samples and direct-injection 'clean' water samples. In Fig. 16.21 the resolved elution profiles of the five coeluted components present in W8 are given. The chromatographic resolution between methalochlor and alachlor in sample W8 was around 0.5. Quantitation of the different components was also rather good (errors in quantitation lower than 10%). A relevant question here is whether the area ratios between elution profiles of the same analyte obtained in the direct injection experiments or in the

preconcentration experiments were reproducible for any analyte. The results showed that the area ratio of the elution profiles was always between 2.3 and 2.7, showing that preconcentration factor was independent of the considered sample, analyte, and/or column. When this ratio was known it could be used in the analysis of new samples. For example, in the analysis of real samples with preconcentration, standard samples at larger concentration prepared in the laboratory can be related with samples injected directly to the column (direct injection). This is the case for instance for the analysis of the augmented data matrix [W8;W1;W2]. Resolution and quantitation of the analytes alachlor and metolachor in W8 was also possible (Table 16.8). Resolved pure spectra of all the five detected components were given in Fig. 16.20.

Similar results were also obtained in the simultaneous analysis of chromatographic runs using the Merck column (Table 16.7). Simultaneous analysis of chromatographic runs obtained using both Waters and Merck column (augmented matrix [W3;W1;W2; M3;M1;M2]) showed that standards obtained with one of the two columns could be also used in the quantitation of the analytes in samples analyzed with the other column. This was true because solvent composition, gradient elution and other physical parameters were kept equal for all the experiments using both columns. Finally, the analysis of metholachlor content in the Delta river water analyzed using the Waters column (matrix W8) was performed using as standard the water sample of metholachlor with no preconcentration using Merck column. Even for this case, a reasonable quantitation of metholachlor was possible with an error of 10% in the prediction of its concentration.

From all these results a stepwise recommended chemometrical procedure is proposed
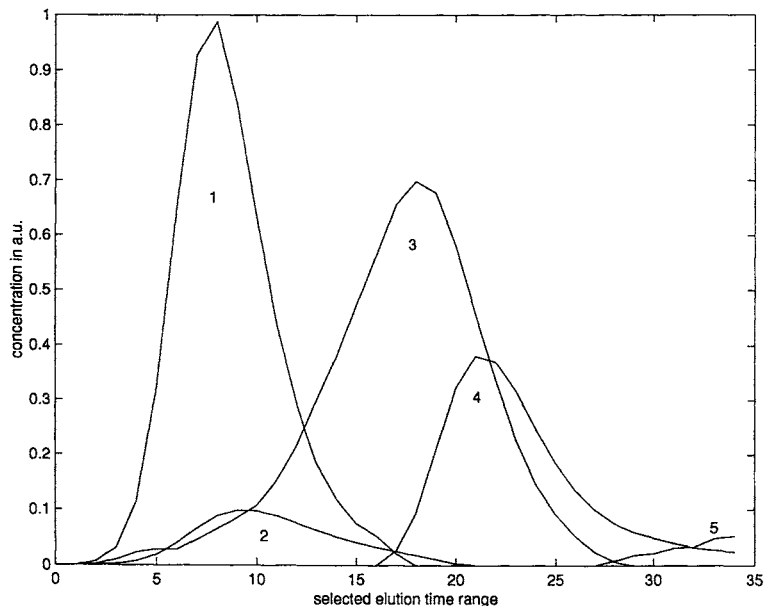
Fig. 16.21. MCR–ALS resolved elution profiles of the five detected components in the simultaneous analysis of data matrix W8 (Ebro Delta river water samples). Identification of the components is the same as in Fig. 16.20. See also Tables 6–8 (reproduced from [78]).

TABLE 16.8

RESULTS (LC–DAD STUDY)

A. Resolutions between metholachlor and alachlor in their mixtures

Matrix	Resolution
W3	0.2
W7	0.4
W8	0.5
M3	0.6
M8	0.7

B. Correlation between species

	1	2	3	4	5
1	1.0000				
2	0.9795	1.0000			
3	0.9992	0.9876	1.0000		
4	0.8788	0.9561	0.9902	1.0000	
5	0.8216	0.9174	0.9681	0.9934	1.0000

for data analysis and quantitation of alachlor and metholachlor in their mixtures at low concentrations.

1. Chromatographic run of the sample having an unknown amount of the analytes alachlor and/or metholachlor to be determined. If they are at very low concentrations, use preconcentration techniques. Storage of experimental data matrix $\mathbf{D_u}$.

2. Chromatographic run of a standard sample of alachlor or metholachlor or of a mixture of both. This standard can be prepared in the lab at higher concentrations avoiding preconcentration techniques. Storage of experimental data matrix $\mathbf{D_s}$.

3. Data pretreatment of matrices $\mathbf{D_u}$ and $\mathbf{D_s}$. Selection of elution time and wavelength range to be included in the analysis. Subtraction of background and solvent contributions.

4. Analysis of the complexity of the data matrix $\mathbf{D_u}$. Determination of the number of coeluted components. Determination of selective ranges for any of the components in any of the two orders of measurement (wavelength or elution time). Multivariate curve resolution of matrix $\mathbf{D_u}$. Resolution of elution profiles and pure spectra of coeluted components. Estimation of the elution order of the coeluted components.

5. Multivariate curve resolution of the column-wise augmented data matrix $[\mathbf{D_u};\mathbf{D_s}]$. Resolution of elution profiles and pure spectra of the coeluted components. Quantitation of analytes in the unknown mixture from the area ratio of resolved elution-concentration profiles in the two data matrices.

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Chapter 17

Application of capillary electrophoresis in environmental analysis

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CONTENTS

17.1 GENERAL INTRODUCTION

17.1.1 Capillary electrophoresis and its position in modern analytical separation science

Electrophoresis and its theory date back to the end of the last century, so one might wonder why it took so long to become the trend in analytical separation science that it is nowadays. Some practical breakthroughs in recent years were prerequisites, and should be mentioned here. Hjertén [1] described the use of narrow-bore glass tubes for zone electrophoresis for the first time in 1967. The inner diameters, however, were still about 3 mm. Mikkers et al. [2] described the use of sub-mm PTFE capillaries (typically 200 μm i.d.) for the separation of both inorganic and organic ions, in 1979. Many authors refer to the important experimental breakthrough described by Jorgenson and Lukacs [3]. Their zone electrophoretic separations were carried out in 75–100 μm i.d. glass capillaries at a voltage of 30 kV. Due to the high field strength and the efficient heat dissipation provided by the capillary format, the separation efficiencies were very high, and are nowadays typically between 100 000 and 600 000 theoretical plates. The introduction of a sophisticated commercial instrument in 1989 [4] was followed by several instruments from other companies in the last few years. A comprehensive reference and textbook, covering all aspects of capillary electrophoresis was published in 1992 [5]. Relevant information can also be found in other books [6–8] and in two reviews [9,10]. One might question the introduction of another high performance analytical separation method. Does it bring applications which cannot be carried out simply by well-established techniques such as capillary gas chromatography (GC) and high performance liquid chromatography (LC)? The most practiced mode, capillary zone electrophoresis (CZE), is used for the separation of ionic and ionizable compounds having sufficient water solubility. These types of analytes are normally not very amenable to GC; at least derivatization will be required. The major competition comes from the LC field (ion-exchange, ion-exclusion and ion-pair chromatography and reversed-phase chromatography). In general, CZE will show much higher plate numbers, shorter analysis times and a different selectivity compared to LC systems. The different selectivity makes CZE complementary to LC. Single peaks in LC might consist of two or mere compounds, as shown by CZE experiment. In this sense, unique separations can be expected. When comparing similar applications of LC and CZE, the latter technique often requires less method development effort, due to its huge separation power and its flexibility and selectivity tuning. In addition, applications on CZE instruments are very economical. No expensive LC columns are needed but very cheap fused silica capillaries, which together with the electrolyte vials, require only a few milliliters of buffer solution.

Nowadays the use of capillary electrophoresis (CE) is a relevant trend in analytical

chemistry, and the number of publications has increased exponentially in recent years at the same time that this technique spreads in the analytical laboratories.

17.1.2 Capillary electrophoresis and environmental analysis

Initially, CE was applied mainly to the field of biochemical analyses, but in recent years its applicability has been demonstrated in all fields of chemical analysis [9,10] including the analysis and purity determination of industrial products [11–13], and the separation of pollutants [14–20]. Despite the impressive CE separations shown at that time, it was concluded in 1990 that CE would not be suitable for environmental analysis unless special injection tricks were to become available [21]. Commercial CE instruments equipped with UV absorbance detectors are able to detect quantities as low as 200 fg and can compete in this regard with the best GC systems. Unfortunately, the injection volumes which can be tolerated in CE, without decreasing the separation performance, are typically a few nano-liters only, which implies a very poor sensitivity as expressed in sample concentration terms. The levels attainable by CE (several hundred ppb) are only found occasionally in environmental samples. Actually, governmental regulations require the determination of lower and lower levels of pollutants in environmental samples (for example, in the EC drinking water directive, that the concentration of any pesticide should be lower than 0.1 ppb) so the concentration sensitivity of commercial CE instruments is far from ideal. Some of these considerations were also valid for the application of capillary GC in environmental analysis. In practice, off-line extraction and concentration techniques are being used to increase the overall concentration sensitivity and, in recent years, special injection techniques have been successfully developed which allow the injection of up to 1000-fold sample volumes, or even entire LC fractions, onto the GC column [22]. In addition, special injection modes have been developed for CE, which allow the introduction of sample volumes as big as the entire volume of the separation capillary [23–35]. A simple clean-up step, followed by a large-volume injection and a high efficiency separation in a CZE system, should be adequate to fulfil the requirements of real-world environmental applications. The determination of phenols, dyes and anionic surfactants were reported as interesting applications.

17.1.3 Scope of this chapter

This chapter assumes some basic knowledge of CE theory and practice. The reader who does not yet have practical and theoretical experience with CE is referred to the book by Li [5].

Section 17.2 describes the different CE modes that are currently available. We show that CE is just the family name and that different CE modes have their own principles and abbreviations. In Section 17.3 different detectors are reviewed, giving emphasis to the mass spectrometric detection. Section 17.4 deals with the special injection tricks that are required for large-volume injections and trace enrichment in capillary zone electrophoresis (CZE). Sections 17.5 and 17.6 are about environmental applications. Finally, conclusions and future trends are given in Section 17.7.

17.2 CE TECHNIQUES

17.2.1 Capillary zone electrophoresis

In the former years of the application of CE to environmental analysis most of the CE work so far was done using the capillary zone electrophoresis (CZE) mode [5,7]. Analytes are separated on the basis of differences in their electrophoretic mobilities, which are related to their charge densities. The separation is carried out in a capillary filled with a continuous background electrolyte (buffer). The direction and the velocity of migration of the analytes are determined by both electrophoresis and electroosmosis (Fig. 17.1). The latter phenomenon originates from ionized silanol groups on the inner wall of the fused silica capillary. They attract positively charged buffer ions which form an electrical double layer. The buffer ions in the mobile region of the double layer will migrate towards the cathode as soon as the voltage has been switched on and induce an electroosmotic flow of the entire liquid in the capillary. In an ordinary fused silica capillary at pH values above 5 or so, the electroosmotic flow will be higher than the electrophoretic migration velocity of
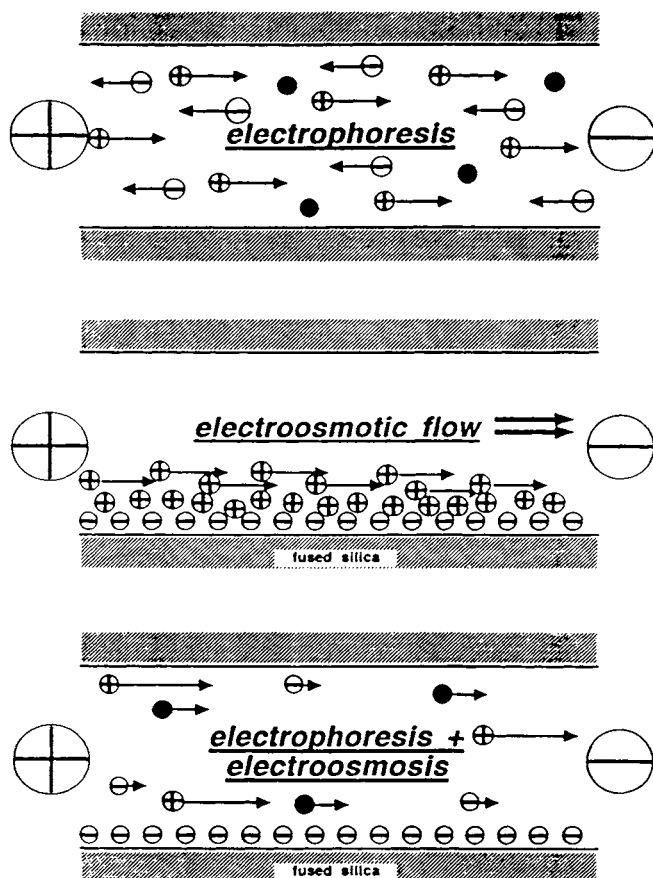
Fig. 17.1. Principles of electrophoresis and electroosmosis a fused silica capillary.

most of the anionic analytes. Consequently, both cationic and anionic compounds will migrate in the direction of the cathode and can be separated within the same run. Contrary to LC, the (electroosmotic) flow might be influenced by sample constituents which interact with the capillary wall. Therefore, reproducibility of migration times is not as good as the reproducibility of the electrophoretic mobilities (i.e. the mobilities after correction for the velocity of the electroosmotic flow). Also, regular integration outputs (mV versus time) are occasionally difficult to compare visually. The most important experimental parameters are the pH, the choice of the buffer and its concentration, and the applied voltage (or current). Its simplicity and similarity to elution chromatography have contributed to the popularity of CZE.

17.2.2 Electrokinetic capillary chromatography

Electrokinetic capillary chromatography (EKC) [5–8] is a subfamily of CE techniques, with the common feature that separations are based on the partitioning of the analytes between two phases having different velocities relative to each other. The most practiced mode, micellar electrokinetic capillary chromatography (MEKC or MECC) was introduced in 1984 [26]. Usually the micelles are charged, and the uncharged analytes partition between the buffer phase (which migrates with the velocity of the electroosmotic flow) and the micellar phase (Fig. 17.2). There are several other options [5]; uncharged micelles with charged analytes, mixed micelles, etc. Other EKC modes are ion-exchange, cyclodextrin-modified and micro-emulsions electrokinetic capillary chromatography.

As compared to CZE, the EKC systems are more complicated because of the additional experimental parameters that have to be optimized. The peak capacity is restricted by the relative narrow elution window which is determined by the velocities of the two phases. In addition, micellar systems are less stable than CZE systems because of the effect of the temperature on the equilibria involved. Despite these drawbacks, EKC has shown separations of neutral analytes with efficiencies as high as in CZE and, moreover, has demon-
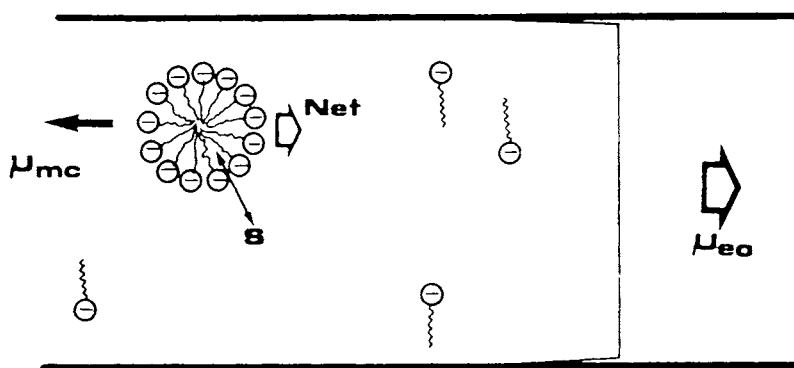
Fig. 17.2. Schematic representation of a system for micellar electrokinetic capillary chromatography (MEKC), showing anionic surfactant molecules, their micelles and the electroosmotic flow profile. S, an uncharged analyte, μ_{eo}, coefficient of electroosmotic flow, μ_{mc}, electrophoretic mobility of the micelle.

strated additional selectivity. This offers a nice alternative for pollutants which are not amenable to GC, and which suffer from the poor efficiency of LC.

17.2.3 Capillary isotachophoresis

Capillary isotachophoresis (CITP) [27] is a CE technique which is carried out in a discontinuous buffer system. The analytes are injected between the so-called leading and terminating electrolytes. A steady-state migration configuration is formed in which the analytes migrate as consecutive zones, which are not diluted by the background electrolyte as in CZE. The concentrating power of CITP may play an important role in trace analysis by CE in general. Contrary to the other CE modes and chromatographic systems, the isotachopherogram consists of a series of steps in which the step heights and the step lengths are indicative, respectively, of the identity and the quantity of the analytes. The current success of CZE will contribute to a revival of CZE will contribute to a revival of CITP, although the different nature of the latter might remain an important instinctive barrier for analytical chemists with a chromatographic background.

17.2.4 Capillary gel electrophoresis and capillary isoelectric focusing

In capillary electrophoresis (CGE) [28], the capillary is filled with a cross-linked gel or with so-called entangled polymers. Separation of molecules are primarily based on differences in their size. Very high plate numbers (several millions) have been realized for biochemical analytes such as oligonucleotides and protein fragments which are normally separated by slab gel electrophoresis. Capillary isoelectric focusing (CIEF) offers separations based on differences in isoelectric points. Both techniques are almost exclusively applied in the biochemical field and seem to be irrelevant for environmental applications.

17.2.5 Capillary electrochromatography

Capillary electrochromatography (CEC) is a new technique for liquid phase analysis. In CEC [5–7], separations are based on partitioning between a mobile and a stationary phase. The combined actions of partitioning chromatography (HPLC) and CE achieve separation of analytes in a liquid sample. [28]. The technique benefits from the flow profile of the electroosmotic 'pump' which is essentially flat, and which leads to smaller plate heights than LC (parabolic flow profile). Since early 1996, the number of reports on CEC in the scientific literature has grown rapidly.

In CEC, a stationary phase packed in a fused silica capillary, id 50–200 μm, is used to obtain separation. The stationary phase is either wall-coated in the capillary or the capillary is packed with LC material, having particle diameters as small as 1.5 μm. Mobile phases typical for RP-type separations, i.e. organic solvent/aqueous buffer mixtures, are used. By the application of an electric field along the axis of the packed capillary, an electro-(endo)-osmotic flow is established, which transports the solutes and the mobile phase through the packed bed. Both neutral and charged compounds can be separated using CEC. When the solutes are uncharged, separation is obtained by differential partitioning of the solutes between mobile and stationary phase. When the solutes to be separated are charged, they will have an additional electrophoretic velocity component which will contribute to separation.

The possibility of using the electro-(endo)-osmotic flow (EOF) as a means to transport solvent through a liquid chromatography column was pointed by Pretorius et al. in 1974 [29]. They used 75–125 μm particles in a 1 mm i.d. glass tube and were able to show that band broadening with electro-(endo)-osmotic flow was considerably smaller than with pressure driven flow. Lately, the studies of Steven and Cortes [30] lead to the conclusion that in capillaries packed with particles <50 μm, EOF velocities were too low to perform efficient chromatography. They concluded that in these packing materials, EOF decreases due to overlap of the double layers. Knox and Grant, however demonstrated theoretically [31] and practically [32] that no double layer overlap occurs with particles down to 1.5 μm in a 0.01 M electrolyte. They could achieve separations of aromatic hydrocarbons in capillaries with 1.5, 3 and 5 μm RP packing materials and with reduced plate heights less than 1.

Concurrently, Tsuda has investigated fundamental aspects of CEC, which were reviewed in 1992 [33]. Further studies on the influence of solvent properties on retention and EOF in CEC were published more recently [34,35].

Several authors have emphasized the necessity of pressurizing the buffer vials with inert gas to 500 psi, to avoid the formation of gas bubbles in the mobile phase at high currents (obtained with buffer concentrations >10 mM) which was also predicted by Knox and Grant [32].

In the first years this technique was not widely applied because of the lack of reproducible packed capillaries, which have been commercialized only recently. Rozing and Dittman reviewed the state of the art in CEC in a review paper late 1995 [36] and since this year, work on CEC has proliferated rapidly. Interesting environmental applications included the determination of polycyclic aromatic hydrocarbons (PAHs) and several pesticide groups.

17.3 DETECTION TECHNIQUES IN CAPILLARY ELECTROPHORESIS

17.3.1 Non-MS detection

The small dimensions employed in CE and the small sample volumes injected present a challenge to achieve sensitive detection without introducing zone dispersion. Zone broadening normally caused by joints, fittings and connectors can be eliminated by on-column detection. On-column UV absorption and fluorescence detection have been the most commonly used detection techniques for CE applications. To achieve on-column UV or fluorescence detection, a window has to be made on the polyamide coating of the fused silica capillary. The simplest way that can be used to form the window is by burning off a small section of the polyamide [37] and cleaning it with acetone or methanol, although alkaline etching [38] and mechanical scraping [39] can also be used. Detection techniques for CE are indicated in Table 17.1.

17.3.1.1 UV-visible detection

UV-visible absorption is currently the most popular detection technique for capillary electrophoresis and related techniques. The main reason for its popularity are its universality, robustness and widespread availability. Fused silica capillary has a UV cut-off

TABLE 17.1

DETECTION TECHNIQUES FOR CE AND TYPICAL SENSITIVITY

Detection technique		Sensitivity (M)
1. UV absorbance detection	(a) On-column UV detection	10^{-6}–10^{-4}
	(b) Axian-beam detection	10^{-8}–10^{-6}
	(c) Use of Z-shaped flow cell	10^{-7}–10^{-6}
	(d) Multireflection detection	10^{-8}–10^{-6}
	(e) Photodiode array or multiwavelength detection	10^{-6}–10^{-4}
2. On-column fluorescence detection	(a) Lamp-based fluorescence detection	10^{-8}–10^{-5}
	(b) Epi-illumination fluorescence microscopy	10^{-12}–10^{-10} or 10^{-7}–10^{-5} with laser or lamp respectively
3. Laser-induced fluorescence detection	(a) On-column laser-induced fluorescence detection	10^{-9}–10^{-7}
	(b) Laser-ind. fluor. with sheath flow cuvette	10^{-12}–10^{-9}
	(c) Fluorimetric photodiode array detection	10^{-7}–10^{-5}
	(d) Use of charged-coupled devices	10^{-12}–10^{-9}
4. Electrochemical detection	(a) Potentiometric detection	10^{-8}–10^{-7}
	(b) Conductivity detection	10^{-8}–10^{-7}
	(c) Amperometric detection	10^{-8}–10^{-6}
5. Indirect detection		10^{-7}–10^{-5}
6. Radioisotope detection		10^{-9}–10^{-7}
7. CE-Mass spectrometry		10^{-9}–10^{-5}
8. Other laser-based detection techniques	(a) Thermooptical absorbance detection	10^{-8}–10^{-5}
	(b) Refractive index detection	10^{-7}–10^{-5}
	(c) Fl. detected circular dichroism detection	10^{-7}–10^{-5}
	(d) Laser Raman detection	10^{-7}–10^{-5}
	Laser-induced capillary vibration	10^{-7}–10^{-5}

around 170 nm and this is suitable for UV detection. The layer of polyamide coating on the outside can be removed to form a detection window as described above. In on-column detection the path length is defined by the inner diameter of the capillary. This limits the sensitivity of absorbance detection techniques, since sensitivity is proportional to path length. Another consideration is that with small capillaries, ideally only the capillary is illuminated during detection in order to reduce stray light.

Some aspects to consider in order to obtain the maximum detection sensitivity (not only in UV-visible but in all absorbance detectors) are the light source used, the design of the signal amplification system, the background light and the optical path length [5]. Some authors [40] have used optical fibers to enhance sensitivity in UV-visible detectors. The optical fibers were positioned on opposite side of the capillary. One fiber was connected to the light source and the other fiber was directed towards a photomultiplier tube for detec-

tion. A Z-shaped flow cell [41] and a multireflection flow cell [42] has been used to enlarge the path length and to improve UV detection with little loss in resolution.

Photodiode array (PDA) detection has also been applied to capillary electrophoresis [43] and commercially systems are now available. The main advantage of PDA detection is that multiwavelength spectral information can be obtained. This spectral information can be used to aid in the identification of unknown compounds, check peak purity and absorbance ratio at different wavelength can be performed to confirm whether there is any overlapping of peaks in the chromatogram.

17.3.1.2 Fluorescence detection

The advantage of fluorescence detectors is that higher sensitivity than for instance in UV-visible absorbance detectors is obtained, however, it is unlikely that this detection technique will be as popular as UV absorbance detection because only a few organic compounds show fluorescent groups in their structure. For this type of compounds pre- or post-column derivatization may be employed to introduce fluorophores to the analyte molecule. The performance of the fluorescence detection for these compounds depends greatly on the design of the pre- or post-column reactor, the stability of the fluorescent derivatives, the effect of the unused derivatizing agents or by-products, the optimum reaction time and the dead volume introduced by the connections. On the other hand, for compounds which exhibit intrinsic fluorescence, or are easy and conveniently derivatized, fluorescence detection will still be the method of choice.

17.3.1.3 Laser-based detection

Lasers are superior excitation sources, which can be used to increase the amount of light focused onto the small detection volume in on-column detection systems. Advantages over arc lamp sources, in both UV and fluorescence detection, include better focusing capabilities which allow the excitation energy to be more effectively applied to very small volumes, and better monochromicity which reduces stray light levels. Laser are particularly useful for sensitive detection on capillaries having inside diameter of less than 50 μm because of the ability to be focused into smaller diameter of less than are possible with arc lamp excitation. The disadvantages are that the wavelengths available from current types of laser sources are rather limited, and that there are possibilities of photodegradation of the analytes caused by the high light intensity.

In particular, laser-induced fluorescence detectors have become very popular in the recent years. The main reason of its growing interest its the sensitivity available with this detection system. The detection of several hundred molecules in CE employing this detector have been demonstrated. Another important aspect of the popularity of this detector is the reduction of the cost, which have permitted to develop commercial systems equipped with laser-induced fluorescence detection. Lasers have also been applied to other detection techniques like thermo-optical absorbance, refractive index or Raman detection, which can provide structural information.

Regarding to the problem of sensitivity in environmental analysis, diode laser-induced fluorescence (DIO-LIF) detection is considered a very promising technique [44]. Diode lasers have several advantageous characteristics: they are small, have a low flicker noise

(<0.05%), and the available wavelengths are in the red (>630 nm) to near-infrared region, where the light scattering and fluorescence background are generally low. Diode lasers emitting at 670 nm have been applied in analytical chemistry, especially in combination of liquid chromatography [45]. Recently, DIO-LIF detection has been combined with capillary electrophoresis [46].

The low linear velocity used in CE is favorable for fluorescence detection because it allows multiple excitation of the analytes during the passage through the illuminated volume; with the picoliter to nanoliter volumes of typical CE flow cells, 100 mW of laser light can already result in an irradiance sufficient for excitation saturation and/or photodegradation of the analyte, which ultimately determines the detection limit. The miniaturization of the detection volume has some disadvantages as well, such as an increased background signal due to scattering and flow-cell fluorescence [47,48]. At longer wavelengths, contributions from these two sources of background are reduced, so that DIO-LIF detection in the red region of the spectrum should be more sensitive than LIF detection at shorter wavelengths. However, because few analytes absorb at wavelengths longer than 630 nm, the direct applicability of DIO-LIF detection is limited. The purpose of this study is to confirm that DIO-LIF detection is very sensitive and that its applicability can be broadened by the introduction of derivatization or indirect detection. Preferably, such measurements are performed at long wavelengths to reduce the interference from fluorescence matrix components.

To summarize, it is clear that diode laser-based spectrometry in general and indirect DIO-LIF detection in particular hold promises, especially for trace analysis in difficult biological matrices where background fluorescence plays an important role. Diode laser-based detection systems capable of measuring very low concentrations of red-absorbing compounds are already available, but the applicability of these devices is limited unless indirect detection is applied. As a result, the chemistry involved is the limiting factor at present.

17.3.1.4 Electrochemical detection

Electrochemical detection provides an alternative strategy to achieve highly sensitive detection. The main problem has been in isolating the detection system from the high electric field in the separation capillary. To date, several approaches have already been shown to be successful, and interest in electrochemical detection methods is expected to grow rapidly. Electrochemical detection systems employed in capillary electrophoresis include methods based on potentiometric measurements, conductivity detection and amperometry. In potentiometric detection, the Nernst potential at the surface of an indicator electrode or across an ion-selective barrier, is measured. In conductivity detection, solution conductivity is measured by placing a pair of electrodes in the capillary and measuring the current passing between the electrodes as a function of potential. In amperometric electrochemical detection the current produced at a fixed potential is measured.

Potentiometric and conductivity detection systems are relatively universal and are usually of low costs. Amperometric detectors are more sensitive and selective. However, the application of this type of technique in the direct mode may be rather limited, since they can be applied to electroactive compounds. By constructing ultramicroelectrodes using semiconductor technology, there should be immense potential in performing multi-

channel detection. In the near future, the development of inert and rugged microelectrodes will probably be an area which is expected to contribute most significantly to the progress of electrochemical detection methods for CE.

17.3.1.5 Indirect detection

The problem of chromophore, fluorophore or electrophore in certain groups of compounds can be overcome by indirect detection methods. Although indirect detection methods are usually less sensitive than their direct counterparts, they provide a virtually universal means for detection. Indirect detection has been successfully applied to UV, fluorescence and electrochemical detection. The main advantage of these modes of detection is that they can be performed using similar instruments than their counterparts and therefore they can be used whenever necessary using available instruments.

17.3.2 MS detection in CE

Among all the available detection techniques, mass spectrometry would probably justifiably regarded as the ultimate tools for detection. The use of mass spectrometry (MS) for detection provides the unique capability to identify unknown compounds. The interfacing of CE to MS has been accomplished by two main types of approaches, namely electrospray ionization (ESI) and continuous-flow fast atom bombardment (CF-FAB). These techniques differ from each other in the method of ion production and transfer form the liquid to the gas phase.

There are also two types of CE–ESI and CE–FAB interfaces, which are based on liquid junction and sheath flow designs. These designs differ by the manner in which the make-up liquid is introduced. The flow rates in CE range from 1 to 100 nl/min, a make-up solvent is needed to support the CF-FAB and ES ionization processes in interfaces operating at 1–50 μl/min. These different types of CE–MS interfaces are discussed in the following sections.

17.3.2.1 CE–CF–FAB–MS

In the CF-FAB interface, a continuous flow of liquid, typically 5–15 μl/min, is delivered through a 10–100 μm capillary to the tip of the CF-FAB probe, which is positioned in the high-vacuum ion source of a mass spectrometer. The FAB–MS technique requires a viscous matrix, typically 5–25% glycerol in different solvents or nitrobenzylalcohol are used as viscous matrix components. This ionization technique has become a common MS method for the analysis of polar, thermally labile or non-volatile compounds. Since CE is also suitable for the analysis of these kind of compounds, interfacing CE with FAB–MS has become an area of intensive research.

In the sheath flow (or coaxial) interface the design is based on the design used for LC–MS (see Fig. 17.3) and the coaxial make-up liquid allows for independent optimization of the composition and the flow rates of the CE capillary effluent and the FAB matrix mixture. Since the CE capillary outlet is positioned in the high-vacuum ion source, the sheath flow design is restricted to small-diameter CE capillaries (10–15 μm i.d.) to avoid excessive vacuum-induced (or pressure-driven) flow. Such pressure-driven flow in the capillary would result in a reduced efficiency of the CE separation because it induces a

parabolic instead of the preferred plug-type flow profile [49]. The small column inner diameter, however, significantly restricts sample loadability and small injection volumes result in poor concentration detection limits. This is a severe drawback in environmental applications, where sample volumes are practically unlimited but analyte concentrations are usually very low.

In a liquid-junction design, the analytical capillary column is aligned with a transfer column (Fig. 17.3). The junction is immersed in the make-up solution. The narrow gap between these two columns (approximately 25 μm) allows the make-up liquid and the analytical capillary effluent to flow into the transfer column, which then delivers the
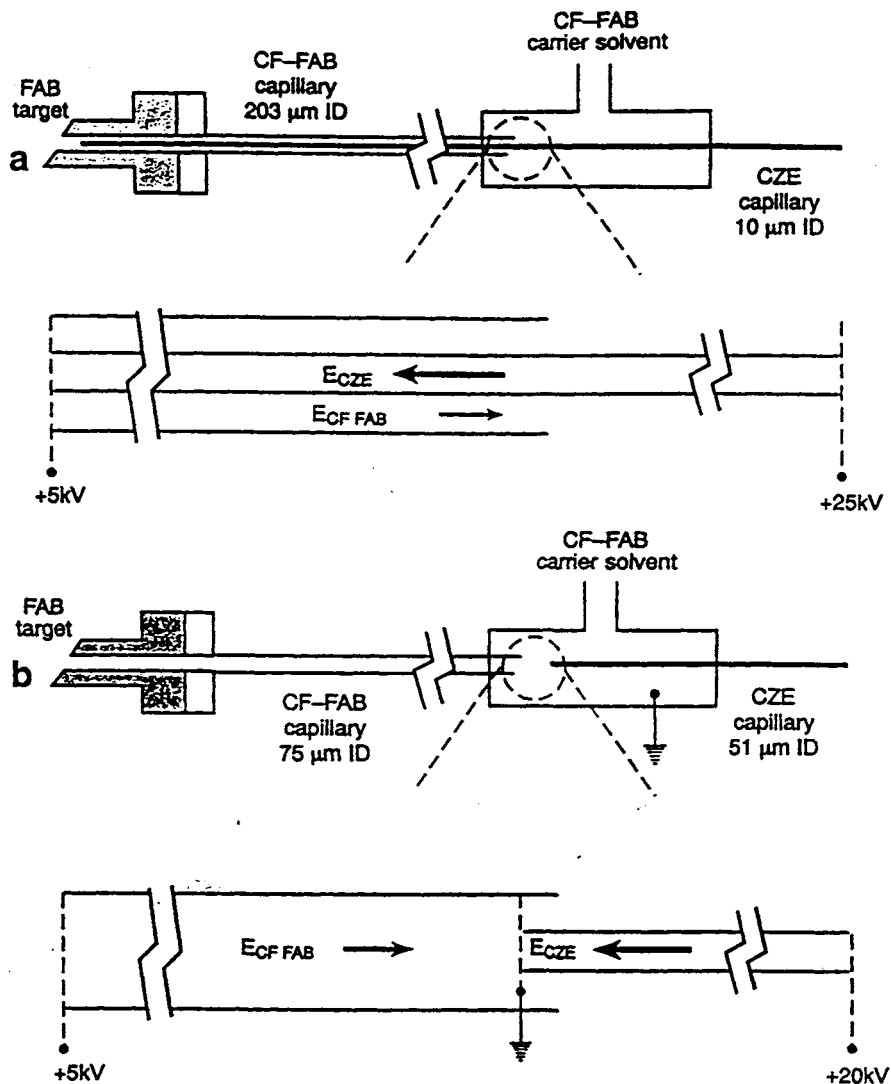
Fig. 17.3. Liquid junction design for CE–FAB–MS interface to CE.

mixture to the tip of the FAB probe. This design of CE–CF-FAB interface was found relatively easy to set up and handle [50], but extreme care was needed in maintaining good separation efficiency and avoiding loss of resolution caused by the interface. Sheath flow and liquid–junction interfaces have been compared [45]. The sheath flow interface showed superior performance with respect to the number of theoretical plates and thus resolution, but it was more difficult to operate due to column plugging problems. In addition, in the sheath flow CF-FAB interface, the low column loadability reduces concentration detection limits.

17.3.2.2 CE–ESI–MS

In an electrospray interface, the analyte solution is electrostatically nebulized in a 3–5 kV electric field where it forms a fine mist of highly charged droplets. Further evaporation of solvent from the droplets is facilitated by a countercurrent flow of heated nitrogen. When the droplet charge-to-size ratio approaches or exceeds the Rayleigh limit, the ionized analyte particles are transferred from solution to the gas-phase. The liquid nebulization process can be aided by pneumatic assistance of a coaxial gas stream and the interface is then called ionspray.

Pleasance et al. [51] compared the sheath flow and the liquid junction interface designs. They were able to change from one configuration to another, although obtaining the correct distance between the capillaries in the liquid-junction system frequently required several attempts. When the gap was too large, the analyte diffused into the fluid in the liquid-junction interface causing deterioration of the electrophoretic resolution. It was concluded that the sheath flow design was more robust and flexible while giving more reproducible results compared with the liquid-junction configuration.

A common problem in CE–MS is that due to the high efficiency of CE, the mass analyzer is not always able to scan fast enough to obtain the adequate number of scans for a given CE peak. This problem can be overwhelmed when a loss in efficiency is produced by the interface or by using a selective ion monitoring (SIM) or selective reaction monitoring (SIR) scan instead of the full-range scan. In this approach, however, the gain in sensitivity may be offset by loss of selectivity of the method because the full spectrum of the analyte is not provided.

Although CE–MS is a relatively new analytical technique and mass spectrometers are generally more expensive than other detectors, there will certainly be a need for such a systems, since they can serve as the definitive method, and they may be indispensable tools in environmental applications for unequivocal confirmation of pollutants.

From the viewpoint of improving detection sensitivity, it should be recognized that in addition to instrumental developments, other approaches can also be adopted to enhance detection sensitivity in CE. Sample preconcentration can be performed during injection by stacking and field amplified sample injection techniques which are discussed in the next section.

17.4 TRACE ENRICHMENT IN CAPILLARY ELECTROPHORESIS

Because extremely small volumes of solutions are injected in CE (from picoliters to tens

of nanoliters), preconcentration, clean-up and on-line sample concentration is often required. A characteristic of environmental analysis, in contrast to biochemical analysis, is the large amount of sample that is usually available for analysis (e.g. lakes, rivers, industrial sludges, agricultural soils, etc.) and the pollutants are normally present in these samples in very diluted concentrations. Thus, techniques of analyte preconcentration used in chromatography, like solvent evaporation, supercritical fluid extraction (SFE), solid-phase extraction (SPE) and derivatization can be used in CE. However, special tricks can also be used with the different CE modes to improve the detection limits by means of field amplification injection techniques and others which are reviewed in the following chapter. These CE techniques combined with the off-line sample concentration techniques common to GC or LC can be a solution to achieve the required sensitivity for environmental analysis.

17.4.1 Field-amplified injection techniques

The field-amplified injection techniques are based on the fact that the electrophoretic velocity of an ion depends linearly on the field strength, i.e. the applied voltage divided by the length of the capillary. When an analyte is dissolved in a sample matrix having a lower conductivity than the background electrolyte (buffer) in the capillary, then this analyte will experience locally an increased field strength and will migrate towards with a higher velocity (proportional to the ratio of the conductivities in the background electrolyte and in the sample matrix). When the analyte reaches the boundary between the sample matrix zone and the background electrolyte, it will slow down again stack into a zone much shorter than the original sample zone, thus the analyte has been preconcentrated or focused on-column. This phenomenon is shown in Fig. 17.4. Samples are often dissolved in diluted buffer or in water. The original sample can be injected either hydrodynamically or electrokinetically. As it can be seen in Fig. 17.4, cationic compounds are focused at the front of the sample zone, and anionic compounds at the back of the sample zone. For the sake of simplicity, the electroosmotic flow gas been ignored so far. The total electroosmotic flow in the capillary will be composed of all local electroosmotic contributions; in the present case, the higher electroosmotic flow in the sample zone and the lower electroosmotic flow in the rest of the capillary. Of course, the influence of the sample zone is not significant when its length is very short compared to the total length of the capillary. However, when one intends to inject a larger volume of sample dissolved in diluted buffer, the following problem arises. A hydrostatic pressure is created by the mismatch between the local electroosmoses on both sides of the sample background electrolyte boundary. Thus, a laminar backflow is created which causes additional band-broadening [52] and restricts the maximum enrichment that can be obtained by a factor of ten.

Fig. 17.5 [53] shows what happens when the sample volume, having a matrix of ten-fold diluted buffer, is increased ((hydrodynamic injection). The peak area increases linearly, but the peak heights show that at larger volumes the enrichment will be counterbalanced by the additional band-broadening. Normally, the detection limit of a pollutant can be as low as 200 ppb, using a UV absorbance detector operating at 200 nm [54]. In a CZE system with a strong electroosmotic flow, which is normally the case, field-amplified injection can improve the detection limit towards 20 ppb. Fortunately, Chien and Burgi [23] showed that the enrichment factor obtainable by field amplification can also benefit

A

B
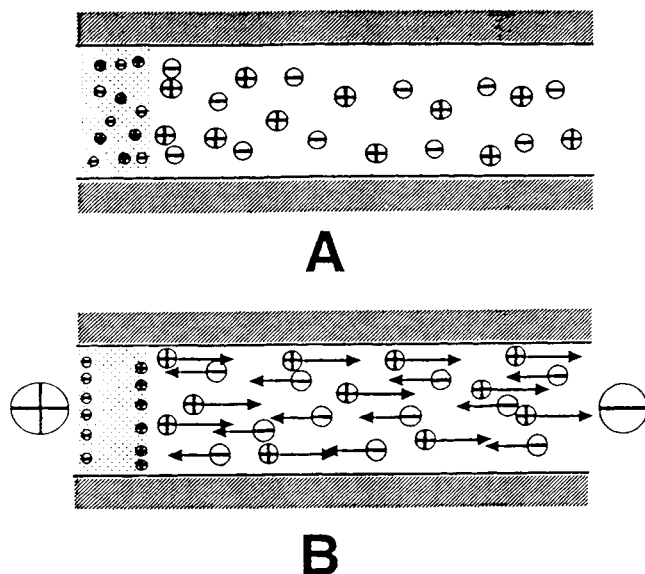
Fig. 17.4. Field-amplified injection. (A) A sample dissolved in diluted buffer, has been injected hydrodynamically. (B) The voltage has been switched on, the analytes within the sample zone experience a higher electric field and move with a high velocity to the boundary with the CZE buffer (focusing). The electroosmotic flow has been ignored for simplicity.

from the electroosmotic flow. Their idea is elegant and simple; remove the relative big sample matrix zone during or after the enrichment step, but before the start of the actual CZE separation, thereby avoiding the problem of the mismatch of local electroosmotic flows. This can be accomplished by pumping out the matrix zone, using the electroosmotic flow.

Fig. 17.6 shows how to proceed for anionic analytes (the enrichment of cationic analytes demands the use of a modified fused silica capillary with a reversed surface charge and an electroosmotic flow in the direction of the anode [55–57]). A relatively large sample volume is injected hydrodynamically; next trace enrichment by field amplification, and pumping out of the sample matrix by the electroosmotic flow, occur at reversed polarity of the applied electric field. Note that contrary to the normal situation (cf. below, in Fig. 17.1), the electrophoretic migration velocity in the sample zone will be much higher than the electroosmotic flow under these field-amplified conditions. Then, at a moment indicated by the value of the current (when the current has reached approx. 95% of its value when no sample zone is present), and the focused analyte zone has almost reached the beginning of the capillary, the polarity is switched back to its original position for the actual CZE separation. Careful monitoring of the current during the trace enrichment step at reversed polarity is essential to avoid loss of the enriched analytes into the buffer vial.

The maximum sample volume that can be preconcentrated in this way, without sample losses caused by initial backflush into the buffer vial, depends on both the electrophoretic mobility of the analyte and the overall magnitude of the electroosmotic flow [23]. Depending on these variables, the injection zones can be as long as one-third of the entire capillary
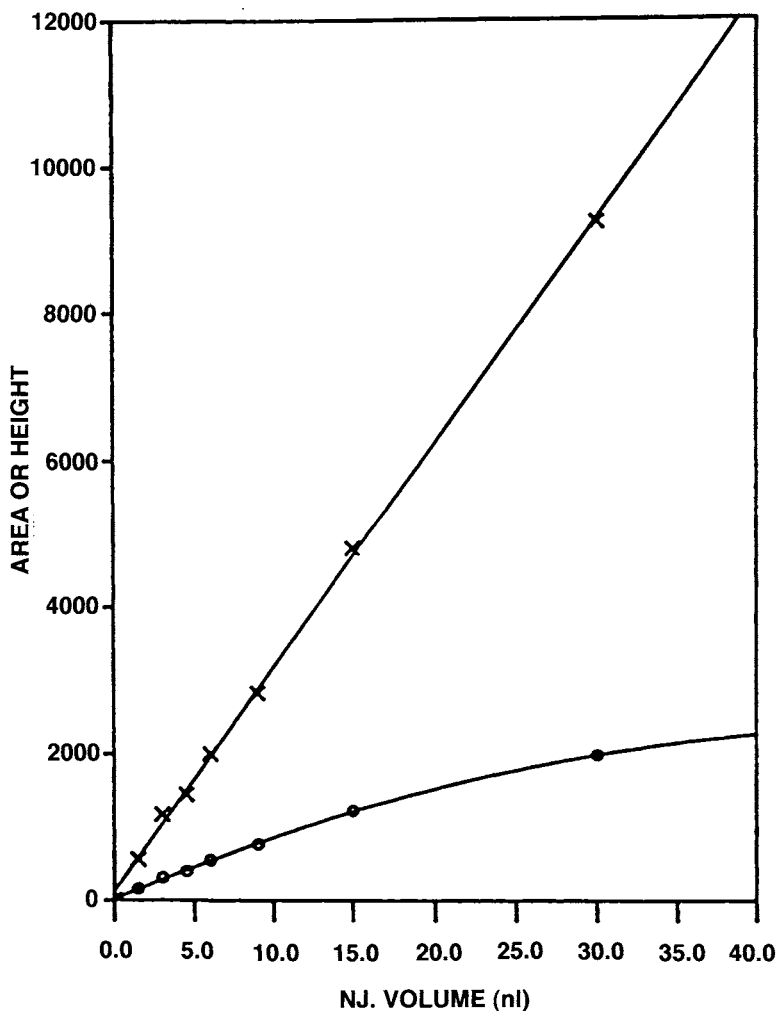
Fig. 17.5. Impact of the sample volume on the peak area (×) and peak height (○) (thus reflecting additional peak dispersion) under field-amplified injection conditions. Fused silica capillary 50 μm × 72 cm i.d.; 40 mM ammonium acetate buffer (pH 4.8); separation at +25 kV. Analyte: *p*-aminobenzoic acid dissolved in tenfold diluted buffer solution.

length, or even more. It has been suggested that one can fill the entire capillary with the sample solution for convenience when large volumes cannot be hydrodynamically injected with sufficient instrumental accuracy and precision, thereby accepting the initial backflush and some sample loss. According to [25], these large-volume injection techniques yield enrichment factors of more than 500, thus allowing environmental analysis at the sub-ppb level. The same technique can be extended to electrokinetic field-amplified injection by replacing the buffer vial by the sample vial during the reversed polarity step.
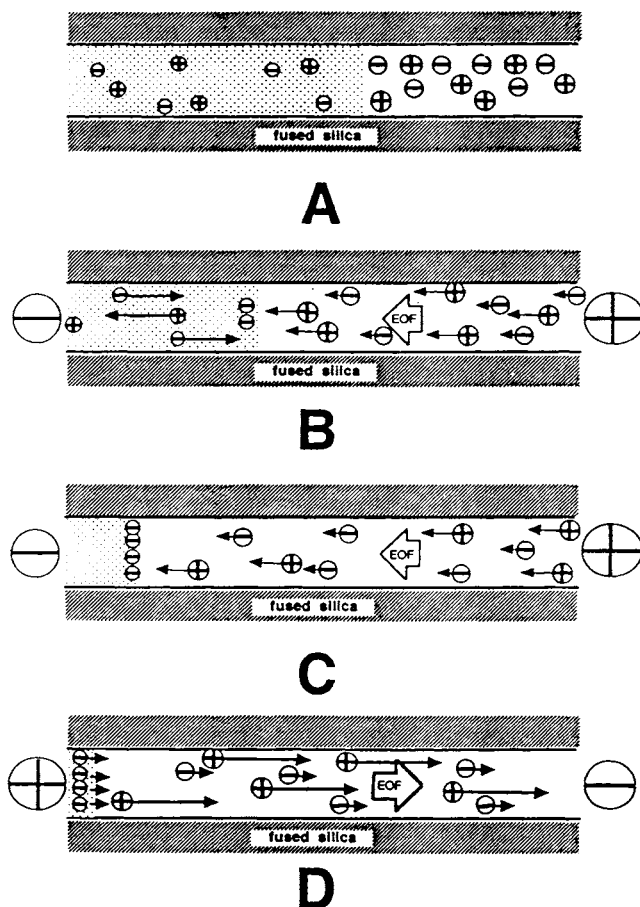
Fig. 17.6. Field-amplified injection of large sample volumes using sample matrix removal by the electroosmotic flow under reversed polarity conditions. (A) A very large volume of sample dissolved in diluted buffer has been injected hydrodynamically. (B,C) The voltage has been switched on and the original sample matrix is pushed back into the buffer vial by the electroosmotic flow. At the same time, anionic analytes in the sample matrix move with a very high velocity to the boundary with the CZE buffer (focusing). (D) The polarity is switched back to its normal position for CZE when the analyte zone has reached the beginning of the capillary.

Of course, some discrimination will occur because of additional electrokinetic injection, but enrichment factors can be as high as 1000.

From the real applications point of view, field-amplified injection requires samples to have a relatively low and reproducible conductivity. It is obvious that environmental sample matrices can be very diverse in nature, so in practice one has to filter the (aqueous) sample in order to avoid plugging and to exchange the original sample matrix for a matrix that meets the above requirements.

17.4.2 Solid phase extraction inside the capillary

Trace enrichment of (environmental) sample in CZE can be performed via solid phase extraction. A short plug of a reversed-phase type packing material can be incorporated at the beginning of the capillary [58] or a hydrophobic stationary phase can be covalently bonded to the inner wall of the capillary [14]. Because of the low amount of the wall-coated stationary phase, the breakthrough volumes and the capacity and hence the enrichment factors are rather limited (10–35-fold) [14]. The capillaries with the plug of reversed-phase material have a somewhat higher capacity and will show higher breakthrough volumes and enrichment (estimation: up to 100-fold). The disadvantages of in-column solids phase extraction should be mentioned. Unlike off-line solid phase extraction, one cannot introduce the raw environmental sample directly because of the risk of plugging the system and suffering severe contamination. In addition, one has to introduce a sample which is, at least partially, cleaned or extracted and should not contain a high percentage of organic solvent. These types of capillaries are only available from a limited number of supplies and are at least thirty times more expensive than a simple fused silica CZE capillary, which can be cut to the desired length and provided with a detection window, using a lighter, in your own laboratory.

17.4.3 On-line coupling of isotachophoresis

Isotachophoresis (cf. Section 2.3) is a technique by which sample zones of low concen-
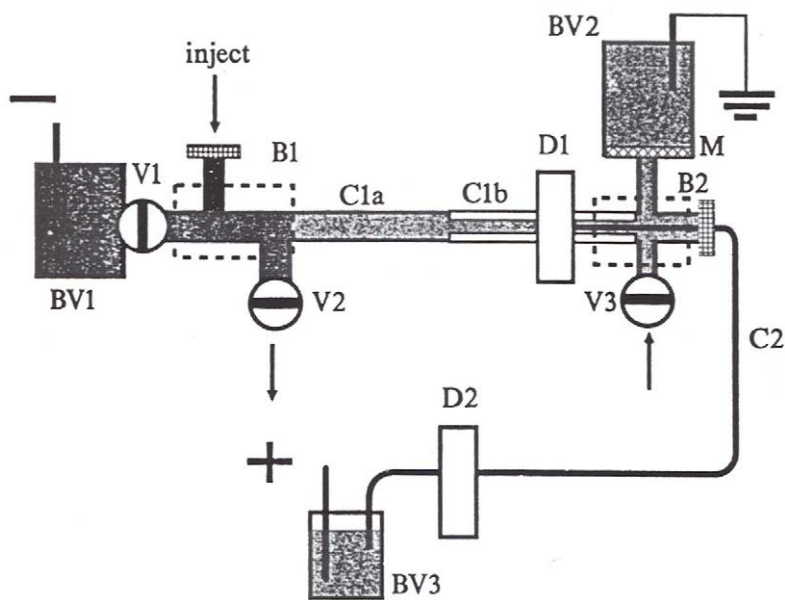
Fig. 17.7. Schematic diagram of an ITP–CZE system. V1, V2, V3, valves; D1, ITP UV absorbance detector; D2, CZE laser-induced fluorescence detector; M, membrane; BV1, terminating buffer vial; BV2, leading buffer vial; BV3, CZE buffer vial; B1 injection block; B2, ITP–CZE interface block; C1a, separation part of the ITP capillary; C1b, detection part of the ITP capillary; C2, CZE capillary.

tration will be focused almost to the concentration level of the leading buffer, following the Kohlrausch regulating function [27]. From the point of view of trace analysis, on-line coupling of ITP and CZE is very attractive [59–61]. A typical example is shown in Fig. 17.7. In practice, one would prefer to inject a heart-cut from the analyte zone, focused and purified by ITP, into the CZE system. However, the focused analyte zone might be very short (a few micrometers) and its ITP migration time will be matrix- dependent, so the timing for the electrokinetic injection into the CZE part of the system will be very critical. Reproducible transfer can be assured only when a larger zone from the ITP is injected into the CZE. Because of the split ratio at the ITP–CZE coupling point, only a part of the ITP zone (according to [56], about 10%) is injected into the CZE system. Nevertheless, using a 10 μl injection on the ITP-part, after on-line ITP–CZE, an enrichment factor of 1000 (as compared to CZE only) can be achieved, with a precision at the 0.5 ppb level of 3.7% rel. SD. Additional selectivity can be incorporated in the ITP part by proper selection of the leading and terminating electrolytes. Certainly, ITP–CZE is an interesting option for environmental trace analysis. Very recently, it has been demonstrated that the rather complex experimental set-up shown in Fig. 17.7, can be replaced by a single-capillary system using a fully a fully automated commercially available apparatus [62,63].

17.4.4 Transient isotachophoresis stage in capillary zone electrophoresis

In regular CZE practice, samples are being dissolved in the background electrolyte (buffer). It has been shown in Section 4.1, that it might be very attractive to dissolve the sample in diluted buffer or water. On the other hand, it might be disastrous when a sample is dissolved in a matrix of very high conductivity because the sample zone may broaden and the separation efficiency and the concentration sensitivity may decrease significantly. There is one exception to this rule; when the sample matrix contains an excess of an ion having an electrophoretic mobility higher than the analytes, and when the buffer contains and ion with an electrophoretic mobility smaller than the analytes. In such a case, the high mobility matrix ion can act as a leading ion, and the buffer ion as a terminating ion, similar to ITP (cf. Section 2.3). The excess of high mobility ions in the sample matrix will be forced through and create a relatively long zone having a concentration adapted to the background electrolyte, following the Kohlrausch regulating function. Similarly, the zone of analytes having a lower concentration will be compressed, so that trace enrichment will occur as in regular ITP.

Beckers and Everaerts described this situation as electrophoresis with 'two leading ions' [64,65]. Unlike the situation in ITP, the background electrolyte is continuous and the ITP-like state will be realized only for a limited period of time (transient ITP), since the zone of high mobility ions will start to migrate following the normal CZE mechanism. The analytes which have a mobility relatively close to the high mobility matrix ion will benefit from a later moment at which the ITP mode changes into the CZE mode, i.e. they will be enriched more than the analytes of relatively low mobility.

An example of transient ITP en the absence of an electroosmotic flow (at low pH) is shown in Fig. 17.8A: a relatively large volume of a sample of *p*-toluenesulfonic acid dissolved in the background electrolyte (10 mM phosphate buffer, pH 2.5), shows severe band-broadening and a distorted peak shape. The same sample dissolved in water shows peak focusing due to the field amplification (cf. Section 4.1). Dissolution in an excess of
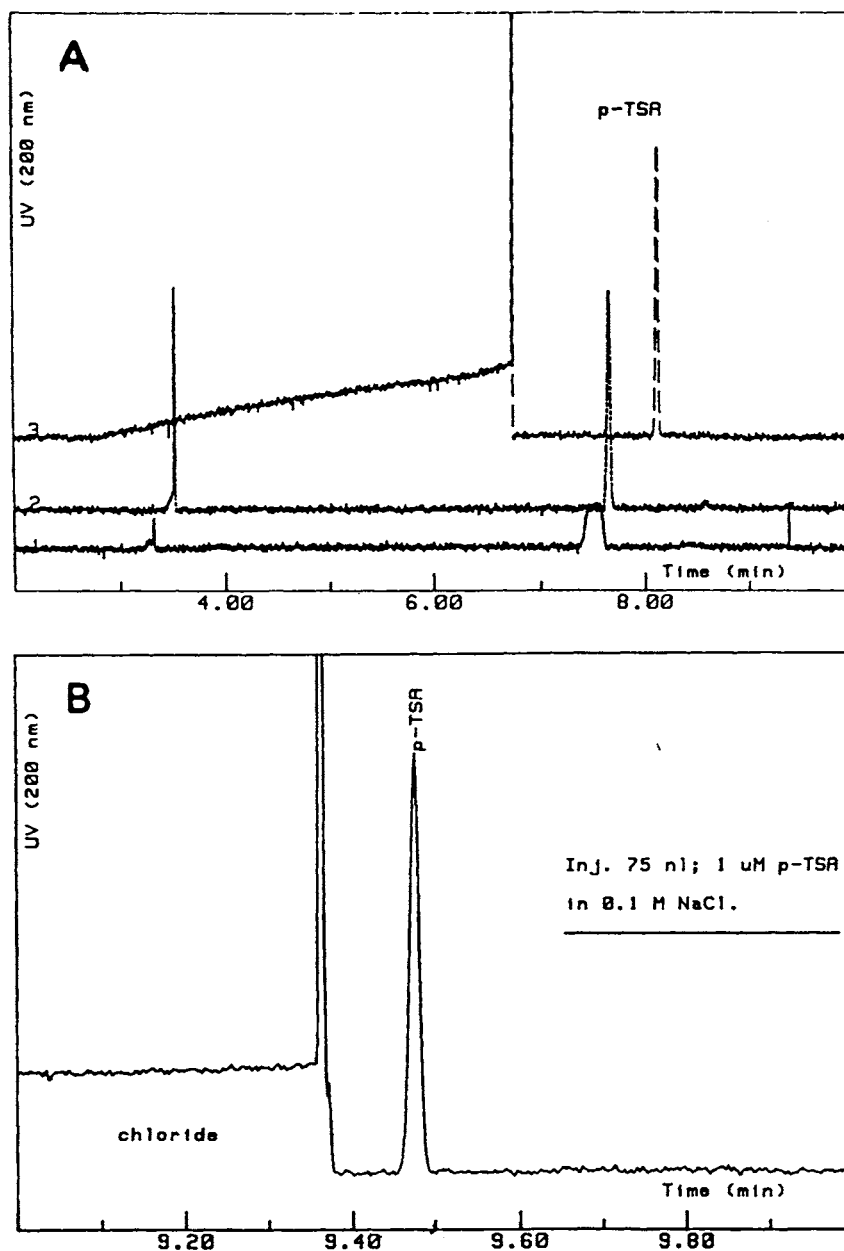
Fig. 17.8. (A) Electropherograms obtained after injection of 15 nl of p-toluenesulfonic acid (10^{-5} M) dissolved in (1) CZE buffer, (2) water and (3) 0.1 M sodium chloride solution. (B) Electropherogram after a transient ITP–CZE run injection of 75 nl (10^{-6} M) in 0.1 M sodium chloride solution. Conditions: fused silica capillary 50 μm × 72 i.d.cm, CZE buffer 10 mM phosphate (pH 2.5) (electroosmotic flow negligible), separation at −25 kV (constant voltage mode).

sodium chloride shows the highest enrichment factor, and an increase in migration time, because of the huge chloride zone which has been forced through. Further optimization of the sodium chloride concentration and the injection volume yields the enrichment as shown in Fig. 17.8B. The analyte migrates close to the chloride zone, and the detection limit has been lowered to 5×10^{-8} M which corresponds to an enrichment factor of about 200. The peak width has reduced considerably and had to be registered at a data sampling frequency of 50 Hz (peak represents 3.7 million theoretical plates). The enrichment can be carried out using an unmodified commercially available CE instrument.

The same principles can be applied to a CZE situation with the existence of a high electroosmotic flow. The major difference is that the sample matrix should contain an excess of low mobility ion and the background electrolyte (buffer) should have an electrophoretic mobility higher than those of the analytes. In such a situation, the excess of low mobility ion will be forced through and its concentration will adapt and the analytes will be enriched and those having the smaller electrophoretic mobilities will undergo the ITP-like process for a longer time. An example is shown in Fig. 17.9A. A 20 mM phosphate buffer (pH 6.3) was used as the background electrolyte and morpholinoethanesulfonate (MES) in excess as the matrix ion. Again, *p*-toluenesulfonic acid dissolved in buffer, water, or an excess of matrix ion, were compared and again the latter showed the highest enrichment factor and a relatively long migration time. Further optimization gives the result of Fig. 17.9B, which shows a peak (impurity) migrating close to the excess of MES, having 13 million theoretical plates, and a second peak having 3.6 million plates and an enrichment factor of about 100. One should be aware of some disadvantages. As in ITP, it may occasionally be difficult to find suitable combination of transient leading ion and terminating ion, the migration times of analytes in unknown samples may be hard to predict, and irreproducibility of the electroosmosis flow will give bad enrichment performance. In addition, it should be recalled that the analytes which migrate close to the matrix ion will take the maximum advantage of ITP stage, so enrichment of analytes with a wide range of electrophoretic mobilities is not feasible. Despite these drawbacks, environmental trace analysis of sea water samples could very well be amenable to CZE with transient ITP, because of the presence of the high mobility saline matrix which can act as a leading ion in CZE separations without electroosmotic flow (cf. Fig. 17.8).

17.4.5 LC–CE preconcentration system

In this case, two capillaries are also coupled; the first capillary is used for the preconcentration process by LC, and the second one for the separation. This type of procedure can deal with high salt concentrations, for example, three benzoic acids were determined in high concentration of sodium chloride using this model [66].

In CE, when the salt concentration is too high, compared with that of the CE electrolyte, the electric field over the sample plug is too low. As a result, the peak sharpening effect is less effective. However, when LC is coupled to a CE system, the analytes have to be trapped on the LC column and the peak shape of the analytes is the same for different salt concentrations, because of the sample cleaning effect of the LC column. Furthermore, the system can also be used for sample preconcentration by loading larger samples volumes on the LC column.
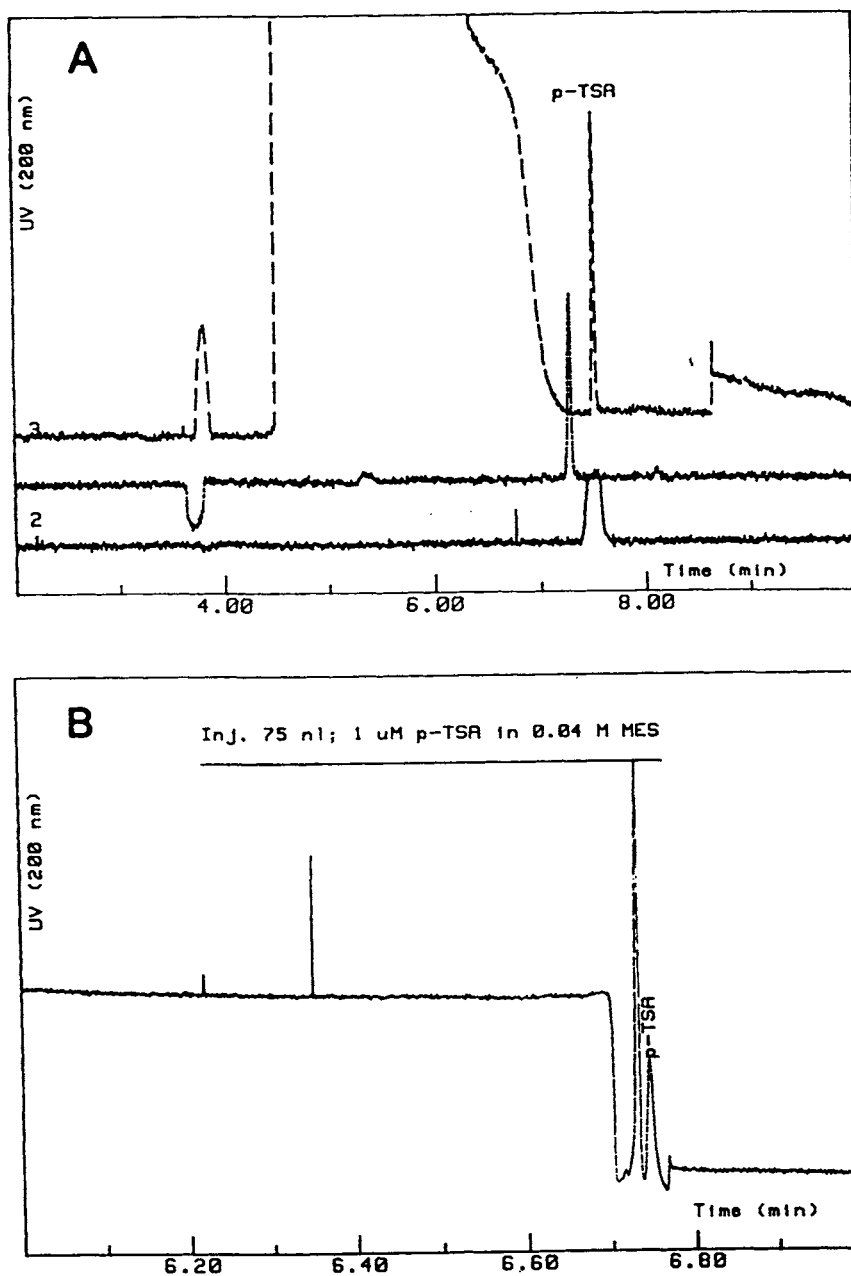
Fig. 17.9. (A) Electropherograms obtained after injection of 15 nl of p-toluenesulfonic acid (10^{-5} M) dissolved in (1) CZE buffer, (2) water and (3) 0.04 M morpholinoethanesulfonic acid solution. (B) Electropherogram after a transient ITP–CZE run, injection of 75 nl (10^{-6} M) in) 0.04 M morpholinoethanesulfonic acid solution. Conditions: CZE buffer, 20 mM sodium phosphate (pH 6.3), separation at +25 kV; other conditions, see Fig. 17.8.

17.4.6 On-line solid phase extraction CE

On-line SPE preconcentration with CE has been shown as alternative to other approaches performed within the capillary. Several works [62–64] have demonstrated the potential of this procedure. The development of membrane preconcentration–CE (mPC–CE) allows samples volumes of about 1–100 μl to be introduced onto the CE capillary. Furthermore, employing a membrane base, impregnated with a suitable adsorptive phase, concentration of analytes can be achieved [67]. For example, for small organic molecules, a polymeric styrene-divinyl benzene copolymer (SDB) impregnated membrane can be used [68], whereas, a C-18 silica-based membrane can be used in the analysis of proteins [62].

This technique can also be employed for sample clean up to remove salts and undesirable sample components, allowing the determination of drugs in biological fluids or the direct injection of complex samples without sample pretreatment, since these compounds cannot be retained on the preconcentration step and can be removed prior the analysis by suitable rinsing steps. The mPC–CE–MS analysis of the neuroleptic drug haloperidol from a urine sample [69] showed that in this process, when small organic molecules are eluted from the membrane in organic solvent (e.g., methanol), analyte stacking occurs, because of analytes are now in a low conductivity solution relative to the separation buffer which leads to a high field strength across the organic zone.

17.4.7 Sensitivity improvement outside CE

Trace-enrichment off-line is usually performed by liquid-liquid extraction (LLE) or solid-phase extraction (SPE) techniques. Recently, a new extraction technique, solid-phase microextraction (SPME) has been introduced. In all cases, the pretreatment steps are clearly separated from the separation.

Solid-phase extraction can be used in conjunction with CZE [65–69]. However, the organic solvent used in the elution step has to be compatible with the electrolyte used in the CE process. In some cases, the elimination of the solvent (for example, by evaporation) followed by the redissolution of the sample with other more suitable solvent, can overcome this requirement.

The determination of sulfonylurea herbicides from marsh waters that contained high levels of potentially interfering compounds using a SPE/clean up procedure and a CE method [70] was been shown. Electropherograms of fortified marsh water samples were not distorted by residual components of the sample matrix and the detection limits for all compounds studied ranged from 0.03 to 0.14 μg/l.

A SPE preconcentration step with a highly crosslinked styrene–divinylbenzene copolymer (ENVI-Chrom P) was applied before CZE and HPLC to determine the 11 priority EPA phenols in a river water [71]. In that case, the sample was preconcentrated 100-fold and a reduction of the matrix effect was observed. Other example of SPE applied prior to CE can be seen in the determination of haloacetic compounds in chlorinated water [72]. The influence of the chlorination disinfection process could be observed comparing both figures. SPE step has also been used as clean up process to determine these compounds in swimming pool waters when electrokinetic injection was used as an on-line preconcentration step [73]. The clean-up of only 25 ml enables the detection of these compounds at μg/l

levels in only 8 min, whereas traditional GC methods require a derivatization step and longer analysis times.

Cela et al. [74] demonstrated that SPE could be used followed by a subsequently sample stacking injection. This procedure was carried out to determine phenol, chlorophenols and nitrophenols at levels below those allowed by international legislation in water from the public supply. In that case, the sorbent used was a crosslinked polystyrene (Isolute Env+). The use of this sorbent concentrated water sample volumes of 1 l in 10 min, with good recoveries (over than 75%) for all the phenols studied. Moreover, the combination of these two process (off-line SPE and stacking injection) allowed the phenols to be resolved in 16 min and determined at concentration below 0.5 μg/l.

Solid-phase microextraction (SPME) has been recently developed and successfully applied in conjunction with gas chromatography [75,76]. The SPME process has two steps: the partitioning of the analytes between the sample matrix and a stationary phase which is coated on a fused-silica fiber, and the desorption of the trapped analytes into the analytical instrument.

Despite the fact that SPME has some advantages over the more conventional extraction techniques, LLE and SPE, very few applications of this preconcentration process in conjunction with CE, to our knowledge, have been reported. The reason of this is that SPME is still a novel technique. However, the miniature nature of the extraction fiber suggest that in an immediate future many SPME/CE separations will be developed. A recent application is the determination of barbiturates and benzoates in urine [77]. In that case, alkaline or neutral compounds were not extracted allowing analysis without interferences. Moreover, the extraction and separation of 10 barbiturates took less than 30 min.

SPME with CE has been applied in environmental analysis to determine polycyclic aromatic hydrocarbons (PHAs) [78]. In that case, it was necessary the use of cyclodextrins (CDs) such as a negatively charged sulfobutyloxy-β-CD (SBβCD) and two neutral methyl-β-CD (MβCD) to separate 16 EPA priority PAHs. The fiber (a coat of poly(dimethylsiloxane) (PDMS) over the glass core) was prepared and used for absorbing these compounds from diluted samples until equilibrium was reached. However, in that case, the absorbed analytes were directly released into the CE electrolyte stream, via an adapter, and electrophoretic separation was carried out. The separation of 16 PAHs was achieved in less than 15 min and the most sensitive detection could be achieved with pyrene at about 8 μg/l, while the highest limit of detection was 75 μg/l for anthracene.

17.5 ENVIRONMENTAL APPLICATIONS

In this section the analysis of several groups of contaminants will be reviewed. Due, mainly, to the fact that sensitivity of CE is not adequate to the low concentration levels of real environmental samples, there are not many publications of determinations of pollutants in real matrices and only high resolution separations of specific pollutants are made. For this reason, special attention will be paid to the works that refer to that kind of analysis and the techniques and protocol to achieve adequate detection limits will be discussed. This section will concentrate on some examples of the use of CE rather an attempt to enumerate the reports on the area of pollutant detection in the different environmental sources.

The most relevant applications are mentioned in Table 17.2.

TABLE 17.2

SUMMARY OF THE APPLICATIONS DESCRIBED IN THIS CHAPTER

Compounds	Separation technique	Matrix sample	Detector	LOD (ppb)	Reference
Hexazianone and metabolites	MECC	Groundwater	UV	0.5–5 μg/l(p)[a]	[80]
Atrazine and deg. products	CZE	Aquatic media	UV	–	[82]
Triazines, carbamates, etc.	MECC	Not environm.	UV	<0.1 mg/l	[83]
Hydroxytriazines	CZE and CIEF	Not environm.	UV	–	[84]
Neutral and ionic herbicides	MECC	Not environm.	UV	–	[85]
Triazines, carbamates, phenoxyalkyl acid, phenylurea, organophosphorous, etc.	MECC	Not environm.	UV	–	[86]
2,4-D, Diuron, propane, atrazine, parathion ethyl	MECC		UV	0.01–0.1 μg/l	[87]
Carbofuran, carbaryl	CZE	Tobacco	UV	50, 15 μg/l	[90]
Dimethyldithiocarbamate	Polymer-coated capillary	Industrial waste waters	UV	1000 μg/l	[91]
Quats	CZE		UV	–	[92]
Quats	CZE		Indirect UV	800 μg/l	[93]
Phenoxyacid herbicides	MECC	Aqueous	LIF	2 fg	[95]
Dicamba, 2,4-D, chlorimuron ethyl	CZE	–	UV, LIF	0.1–1 μg/l	[96]
Chlorophenoxyacid herbicides	Enantiomeric	Water	UV	1 (p) μg/l	[97]
Dichloprorp	Enantiomeric	Water	UV	–	[98]
Acid herbicides	Enantiomeric	Soil extract	UV	5×10^{-7} M	[101]
Dichlorophenols	MECC	–	UV	–	[102]
Chlorophenols	MECC	Soil extracts	EC	1 nM	[103,104]
Phenolic acids	CZE	Alcoholic drinks	UV	–	[105]
Sulfonated azo dyes	MECC	Not environm.	UV	–	[106,107]
Sulfonated azo dyes	MECC	–	UV	–	[108]

TABLE 17.2 (*continued*)

Compounds	Separation technique	Matrix sample	Detector	LOD (ppb)	Reference
Sulf. azo and xanthene dyes	MECC	Food	UV	–	[109]
Food colorants	CZE	–	UV	3000 µg/l	[111]
Sulfonated azo dyes	MECC	Water samples	UV	10–150 µg/l	[112]
Food colorants	CZE	Soft drinks	UV	11–300µg/l	[113]
Reactive dyes	CZE	Water effluents	UV	–	[114]
Reactive dyes	MECC	Cotton and wool	UV	–	[115]
Reactive textile dyes	CZE	Sewage effluents	UV	–	[116]
Fatty alcohol ethoxylates	CZE	–	UV after derivatization		[117]
Haloacetic acids, phenols, herbicides	CZE	–	UV	Low ppb (p) µg/l	[73,121]
Sulfonylurea pesticides	CZE	–	CF-FAB–MS	–	[122]
Quaternary ammonium salts	CZE	–	ES–MS	fM–aM	[123]
Paraquat and diquat	CZE	–	ES–MS	–	[124]
Chlorinated acid herbicides and phenols	CZE	Water	ES–MS		[125]
Triazine herbicides	Partially filling MECC	–	ES–MS		[126]
Sulfonylureas	CZE	–	ES–MS	–	[127]
Alkylsulfate detergent, phenoxyacid herbicides, alkylphosphate and impurities	CZE	–	ES–MS	pg range	[128]
Synthetic dyes	MECC	Water and soils	UV		[129]
Azo dyes and sulfonic acids	CZE	–	CF-FAB–MS	–	[130]
Sulfonated azo dyes	CZE	–	ES–MS	High µg/l–low mg/l	[132,133]
Sulfonated azo dyes	CZE	Industrial effl.	ES–MS	100–800 (p) µg/l	[134]
LAS	CZE	WWTP effluents	ES–MS	–	[136]
Aromatic sulfonates	CZE	River and seepage water	CZE-UV CZE-Fluorescence	–	[137]

[a] (p), LOD with preconcentration.

17.5.1 Pesticides

The improvement in detection limits of the analytical methods for the determination of herbicides and pesticides in general and their metabolites, is evidenced by the extensive literature that appears each year [79–88]. The new goals in pesticide analysis are to maximize the detector response for the compounds of interest and to minimize the response for interferences, controlling the analysis time and labor costs. In this respect, new approaches based on progress in instrumental equipment must be used and the traditional tools must be employed in order to check and estimate the real share of a new analytical method. The application of CE to the separation and determination of pesticides is rapidly expanding, although there are until now only limited reports in the area of pesticide detection.

17.5.1.1 Triazines

Triazines are among the most important selective herbicides. Atrazine is the main representative of the triazines being the most used herbicide in USA in 1993 and it has been forbidden in Germany since 1991 because of environmental pollution. Determination of hexazinone and its metabolites in groundwater was possible with a MECC method and a running buffer consisting on 50 mM SDS, 12 mM sodium phosphate, 10 mM sodium borate with 15% methanol [80]. A chromatogram of the separation of the studied compounds of this work is shown in Fig. 17.10. Preconcentration was performed with solid-phase extraction and quantification using UV detection was achieved at levels ranging from 0.5 to 5 ppb. A comparison was made between this CE method and an established HPLC method of the hexazinone and one of its metabolites, resulting in a good correlation of the results. However, analyzing surface waters by HPLC has been a problem because of humic substances which can interfere with the analysis while there appears to be no problem with humic acids when analyzing hexazinone in surface water by CE. This fact has been explained in another work for the determination of atrazine and its metabolites the hydroxytriazines [81]. In CE, these compounds are separated as cations while common interfering substances in environmental waters like humic acids are separated as anions and for this reason no interference was noticed. Owing to this fact the atrazine and its degradation products could be determined without clean-up procedures, which simplifies the analytical protocol. A 50 mM acetate buffer at pH 4.65 was used for the separation which could be achieved in less than 5 min. In addition, the CZE method was used to study the degradation pathway of the atrazine in several aquatic media, combined with other detection methods like LC–TSP–MS. The same author has studied the effect of pH of the buffer on the separation of several classes of triazines [82] and concluded that the optimum pH is observed at pH $= pK_a$ or pH $= pK_{a, min} - 2$ (where $pK_{a, min}$ is the smallest pK_a value of all analytes).

Optimization of the different parameters of a MECC method for the separation and determination of triazine and other kinds of pesticides (carbamates and organophosphorus compounds) has been made [83]. The separation was optimized as a function of pH, surfactant character and concentration and organic modifier. Quantitative aspects were also examined. A determination of the compounds was possible up to the range of 0.1 mg/l
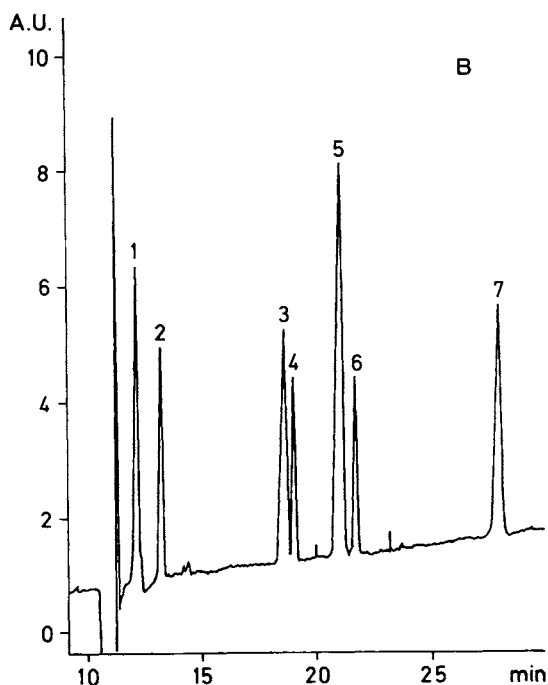
Fig. 17.10. Electropherogram of the separation of hexazinone and metabolites. Analysis conditions: 48.5 cm × 75 μm i.d. capillary column; hydrodynamic injection (12 s at 60 mbar); 50 mM SDS, 12 mM sodium phosphate, 10 mM sodium borate and 15% methanol (pH 9.0) time 0 at 25 μA to 55 μA for first minute and then constant at 55 μA, detection wavelength 225 nm. Peaks: 1 = metabolite C; 2 = metabolite A1; 3 = hexazianone; 4 = metabolite E; 5 = metabolite B; 6 = metabolite D; 7 = atrazine.

and best results were achieved using borate/SDS buffer, pH 8. At a voltage of 20 kV, the investigated compounds were successfully resolved within 13 min.

CZE and CIEF have also been used to separate and determine simultaneously the pK_1, pK_2 and pI values of 12 environmentally relevant hydroxytriazines and the effect of the substituents on the measured values was investigated [84]. Knowledge of the pK and pI of these compounds is important for an understanding of the binding mechanisms of these molecules in environmental matrices. A CIEF method was developed to measure the pI of these compounds and were compared with the values measured by a CZE method, and both techniques agree. pK values for the hydroxytriazines were measured also with CZE methods.

17.5.1.2 Sulfonylureas and phenylureas

The effect of the addition of aliphatic alcohols on the separation by MECC of a mixture of neutral and ionic herbicides (five phenylureas and four phenoxyalkyl acids) have been investigated [85]. Different aliphatic alcohols (from methanol to 1-heptanol) were added
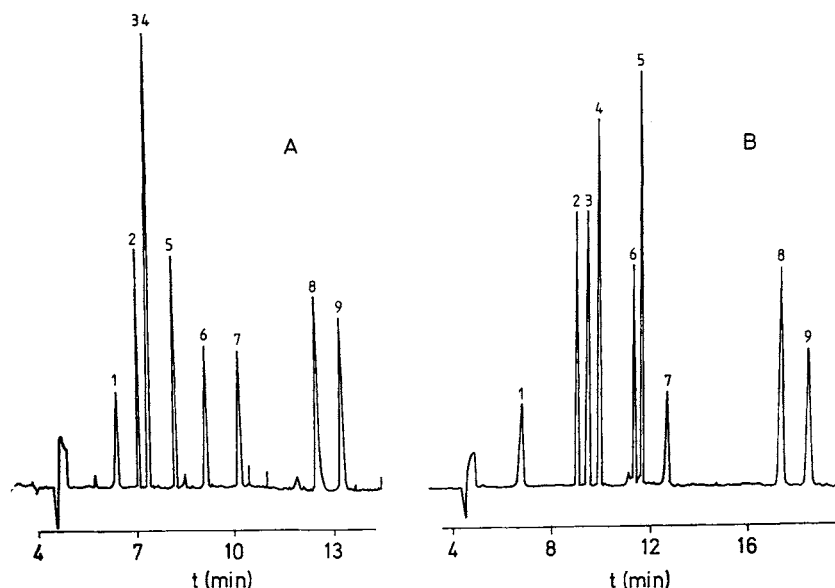
Fig. 17.11. Chromatograms of a mixture of the nine herbicides. Conditions: 0.02 M phosphate buffer, pH 7, 0.05 M SDS, applied voltage 15 kV, $\lambda = 205$ nm. Chromatogram A: no organic modifier. Chromatogram B: 0.38 M (3.5%) of butanol. Peaks: 1, fenuron; 2, Diclorprop; 3, 2,4-D; 4, 2,4,5-T; 5, 2,4-DB; 6, Monuron; 7, Monolinuron; 8, Linuron; 9, Diuron.

to a phosphate buffer (0.02 M) containing 0.05 M SDS as surfactant. The conclusion is that improvement of resolution occurs for anionic herbicides when aliphatic alcohols are present, while separation of neutral compounds seems to be independent of the organic modifiers. This can be seen in Fig. 17.11, where the separation with and without addition of organic modifier is shown. So, when neutral herbicides have to be analyzed the use of these organic modifiers may not be necessary. Furthermore, it has been experimentally found that the optimal alcohol concentration decreases as chain length increases. The addition of *n*-butanol has been used by the same author for the determination of a complex mixture of several pesticides (phenylurea, triazine, carbamate, phenoxyalkyl acid and organophosphorus pesticides with MECC) and compared in terms of efficiency and sensitivity with an HPLC method [86]. When mixtures of ionic and neutral compounds have to be analyzed, MECC is the best solution and when only neutral compounds have to be separated HPLC performs better. Although sensitivity of the HPLC method was 3–5 times higher than that of MECC, in both cases the use of preconcentration prior to injection was needed to achieve detection limits adequate for environmental analysis. To increase detection limits in both methods off-line SPE was applied and in MECC it was combined with a field-amplified injection technique which achieved an enrichment factor between 100 and 200. Other works have been carried out combining off-line SPE and sample stacking [87] (field-amplified injection) and the dependence of this technique on different parameters (like electrophoretic and electroosmotic velocity, polarity of the analytes, injection time applied voltage during stacking process, etc.) has been investigated. Employing a two-step

enrichment process, detection limits were improved (between 0.01 and 0.1 ng/ml) and the sample volume for SPE, and analysis time was reduced.

MECC methods for the analysis of difficult compounds by HPLC like sulfonylureas in waters and their metabolites formed during hydrolysis [88] as well as for the determination of primisulfuron and triasulfuron fortified in environmental waters and soils [89] have also been developed and showed the potential of this technique for their determination.

17.5.1.3 Carbamates

Carbamates comprise an important group of pesticides used as insecticides mainly and noted for their relatively short persistence in the environment. This characteristics have been the reason of their broad use, which have made that these pesticides are now found in several environmental matrices. HPLC is the favored technique for determining carbamates in environmental matrices due to the fact that GC is not amenable to these polar and unstable compounds at the injector temperatures. CE has also been applied recently for the separation and determination of carbamates in several matrices. A method for the analysis of Carbofuran and Carbaryl residues in tobacco samples has been developed combining CZE and UV detection [90]. Two extraction methods were compared: solid-liquid extraction (SLE) and supercritical fluid extraction (SFE) with CO_2-acetone. CZE was efficient for carbamate residue analysis, allowing separation of these pesticides from tobacco compounds, giving good sensitivity with a quantification limit of 0.05 mg/l and 0.015 mg/l for Carbofuran and Carbaryl, respectively. Additionally, SFE combined with CZE gave advantages with respect to time solvent economy and, compared to SLE, SFE showed better capacity of extraction of unstable thermal compounds as carbamates and higher extractive power, establishing an alternative to residue analysis.

The determination of anionic species like sodium dimethyldithiocarbamate in industrial waste waters was performed by CE with direct UV detection [91]. The application of new technology sulfonic acid polymer-coated capillary columns achieved sensitive analysis with robust electroosmotic flow (EOF), where other coated columns and conventional fused-silica had failed due to analyte adsorption problems. Optimum conditions for the separation of cationic, neutral and anionic interferences, using a run buffer solution of 25 mM sodium phosphate, pH 6.5, were obtained. Quantitative analysis were also carried over, with detection limits at 1 mg/l for typical sample size (2 ml) without preconcentration of the analyte. The accuracy of the method was calculated to be better than 95% within targeted values in the working range of the calibration curve.

17.5.1.4 Quats

Quaternary ammonium ion herbicides paraquat (PQ), diquat (DQ) and difenzoquat (DF) which present some shortcomings and do not allow the simultaneous separation of the three compounds in a single sample, have also been analyzed by CE techniques [92], as can be seen in Fig. 17.12. A CZE method with an acetate buffer at pH 4 and UV detection was used and the effect of several parameters, like temperature, pH and buffer cation, on resolution was investigated. The influence of the buffer cation was found to be very important and showed a large effect on resolution between PQ and DQ because it prevents adsorption on the silica capillary surface. Sodium was the best choice for good separation
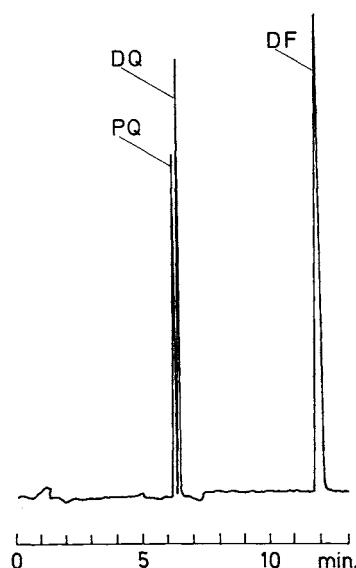
Fig. 17.12. Typical electropherogram of standard solution: PQ, 51.2 mg/l, DQ, 60.0 mg/l, DF, 54.5 mg/l. Electrolyte running solution, acetate buffer, pH 4.0 with 100 mM NaCl; sample injection mode; hydrodynamic, 1 s; applied voltage: +15 kV; temperature, 30°C.

of the three analytes. For the non-absorbing quaternarium ammonium herbicides indirect UV detection methods have been developed [93]. Detection limits of 0.8 mg/l could be achieved using 1-(4-pyridyl)pyridinium chloride hydrochloride, which was the most appropriate of the chromophores compared for indirect detection.

As it has been said before, ITP preconcentration can also be performed using a coupled-column arrangement. The potential of this technique for environmental trace analysis of herbicides such as diquat and paraquat and other polar pollutants has been shown [94].

17.5.1.5 Acidic

MEKC (phosphate buffer, pH 7 with SDS and urea) with LIF detection was used for the trace analysis of phenoxy acid herbicides [95] in order to overcome the poor sensitivity of on-column UV detection. A derivatization procedure was developed which is suitable for nanogram amounts of organic acids. The acids are activated by hydroxybenzotriazol and diisopropylcarbodiimide and reacted with 5-(aminoacetamido) fluorescein in dimethyl-formamide at ambient temperature. A 480 nm Ar laser line was used for excitation. The high efficiency of CE allows the separation of the fluorescein derivatives of all target compounds in a single run. Detection limits of 2 fg can be achieved for a 4 nl injection, but for practical reasons, a minimum of 1 ng per compound should be subjected to the derivatization. The applicability to the method to the extract of an aqueous sample was demonstrated. However, the derivatization procedure is not as robust as some well-established procedures. Extracts of real samples may contain compounds that enhance the
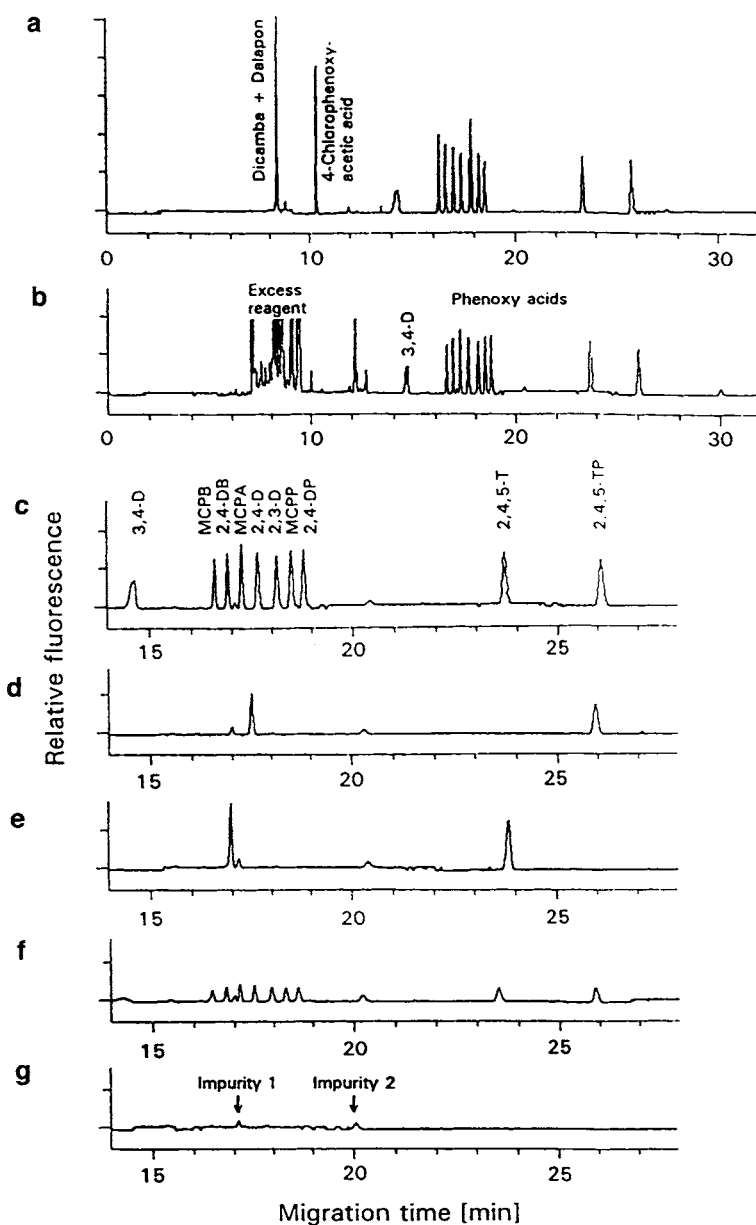
Fig. 17.13. Chromatograms from trace analysis of phenoxy acid herbicides by MEKC with LIF detection after derivatization of stock solutions. Analytical conditions: voltage, 30 kV; capillary, 47 cm × 50 μm i.d.; buffer, 39 mM phosphate pH 7.0, 46 mM SDS, 1670 mM urea, 22.2% (v/v) methanol. (a) Artificial mixture of acid derivatives prepared at a semi-preparative scale; (b) derivatization of a mixture containing 100 pmol per phenoxyacid; (c) enlargement of the relevant part of chromatogram b; (d) derivatization of 2,4-D and 2,4,5-TP, 100 pmol each; (e) derivatization of 2,4-DB and 2,4,5-T, 100 pmol each; (f) derivatization of a mixture containing 20 pmol per phenoxy acid; (g) blank derivatization without phenoxy acids.

decomposition of the derivatizing reagent, thus being needed a modification of the method. An example of CE analysis performed with this method is shown in Fig. 17.13.

CE–UV and CE–LIF have also been used for determining several herbicides that have widespread use in USA [96]. Particularly, Dicamba, 2,4-D and chlorimuron ethyl were analyzed simultaneously by CE–LIF and CE–UV. Dicamba and 2,4-D were derivatized with 4-bromoethyl-7-methoxycoumarin and chlorimuron ethyl was derivatized with dansyl chloride following hydrolysis. Detection limits that in CE–UV are in the range between 0.1 and 1 µg/l for these compounds, with CE–LIF improve up to 10 ng/l.

A CE method was developed involving cyclodextrins to separate seven chlorophenoxy acid herbicides and their enantiomers [97]. The cavity size and the concentration of cyclodextrins heavily influenced the migration time of individual herbicides. The peak resolution also varied with the concentration ratio of cyclodextrins. The optimum experimental conditions were found at 4 mM α-cyclodextrin and 1mM β-cyclodextrin. The studied herbicides could be separated within 7 min; meanwhile, two pairs of enantiomers could also be resolved. Calibration curves for quantification were all above 0.996 and the dynamic range for all the herbicides were at least two orders, from 1 to 100 ppm. By combining with a C_{18} disk preconcentration method, detection limits in the environmental water were below 1 ppb.

Another application of CE analysis with cyclodextrins is the study of the enantiomeric selectivity in the degradation of the chiral herbicide dichloprop [98]. It was observed that the S-($-$)-isomer degraded significantly faster ($t_{1/2} = 4.4$ days) than the R-($+$)-isomer ($t_{1/2} = 8.7$ days). This was contrary to other published studies [99,100]' that show selective degradation of the R-($+$)-isomer by marine microorganisms and soil bacterial cultures. CZE allowed the analysis of soil samples with a simpler and less time-consuming procedure than with GC or HPLC. Furthermore, humic substances and other naturally occurring organic macromolecules appear to interfere less with CZE. The major problem encountered was the variation in migration times, which could be tolerated by adding an EOF marker to track the changes and by spiking aliquots of the sample matrix with a standard.

Other chiral selectors have been used [101], like vancomycin, for the analysis of several acid herbicides, such as aryloxypropionic acids, aryloxyphenoxypropionic acids and an aminopropionic acid derivative, flamprop (see Fig. 17.14). Due to the strong UV absorption of the chiral additive at low wavelength, the partial filling method, which allowed to keep the detector path free of absorbing vancuomycin, was used. The concentration of chiral selector, the capillary temperature and the pH of the background electrolyte strongly influenced the enantiomeric resolution and the selectivity of the separation of chiral herbicides. The best results were achieved with 6 mM vancuomycin, pH 5 at 25°C. The optimized method was tested for the qualitative analysis of a soil extract. Detection limits were 5×10^{-7} for each enantiomer. This method was found to be rapid and cheap compared to other ones, like GC or HPLC where expensive chiral stationary phases or derivatizations are necessary.

17.5.2 Phenols

CE is also a powerful separation technique for the analysis of phenols, as was demonstrated by Ching-Erh Lin and coworkers [102]. In this work, the migration behaviour and selectivity of isomeric dichlorophenols (DCPs) were investigated with MEKC and SDS in
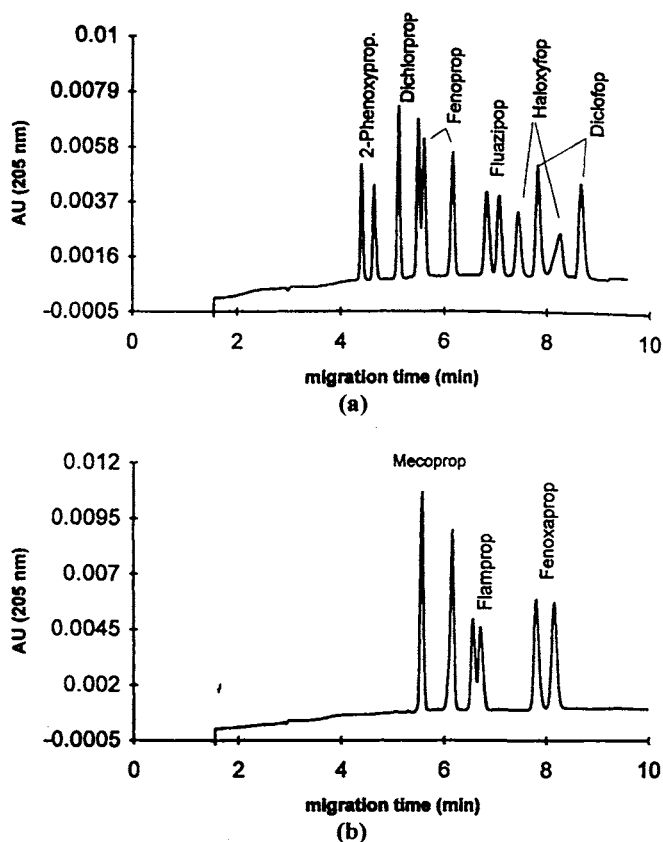
Fig. 17.14. Electropherograms of the enantiomeric separation of herbicides. (a) 2-Phenoxypropionic acid, dichlorprop, fenoprop, fluazifop, haloxyfop and diclofop; (b) mecoprop, flamprop and fenoxaprop. 6 mM of vancomycin partial filling at 34.475 kPa $\times$ 30 s; applied voltage, 20 kV, 47 μA; analyte concentration was 10^{-4} M, with the exception of fenoprop and diclofop (5×10^{-5} M) and 2-phenoxypropionic acid (2×10^{-4} M).

a phosphate–borate buffer solution with UV detection. Micelle concentration and buffer pH are were the two most important parameters affecting the migration times and selectivity as can be seen in Fig. 17.15. A comparison of these factors with CZE was made. By MEKC, the complete resolution of the six DCPs isomers was achieved with [SDS] = 10 mM, at pH 6.00 within 8 min, whereas [SDS] = 30 mM was required at pH 8.14. Conversely, the complete separation of DCPs by CZE is impossible at pH < 6.7. Therefore, this work demonstrates that MEKC is a powerful and versatile separation technique for DCPs isomers.

Widespread applications of CE have been limited by the lack of sensitivity of these techniques. This can be overcome by an amperometric detection cell coupled to a CE system, as shown by some authors [103] for the analysis of chlorophenols. The detector assembled with a gold electrode achieves a sufficient sensitivity in analyzing liquid extracts to enable determination of pentachlorophenol (PCP) in soils at 35 mg/kg. The
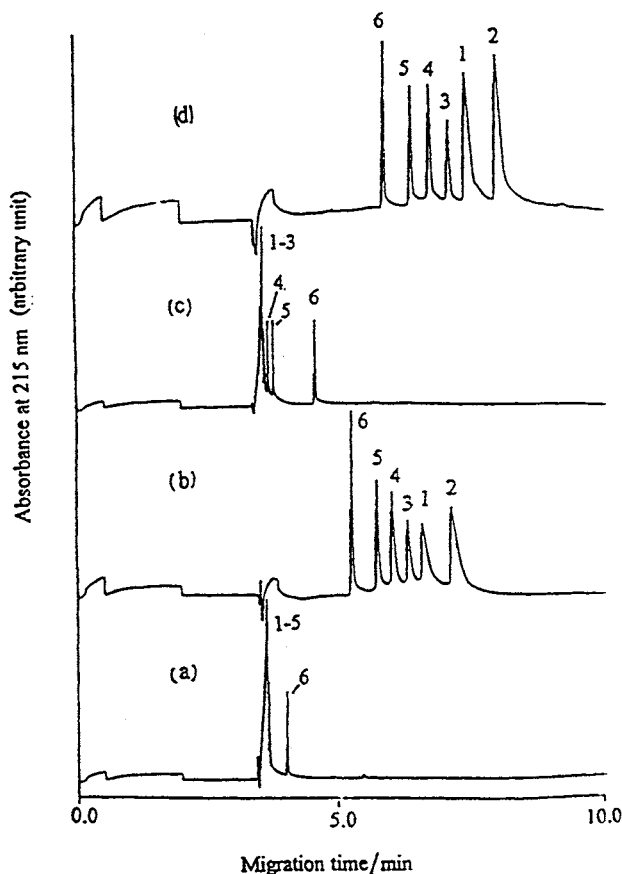
Fig. 17.15. Electropherograms of dichlorophenols at (a) pH 6.00, 0 mM SDS, (b) pH 6.00, 10 mM SDS, (c) pH 6.5, 0 mM SDS, (d) pH 6.5, 15 mM SDS. Peaks: 1 = 3,4-DCP; 2 = 3,5-DCP; 3 = 2,4-DCP; 4 = 2,3-DCP; 5 = 2,5-DCP; 6 = 2,6-DCP. Operating conditions, 15 kV, 25°C; capillary, 44 cm × 50 μm i.d. fused silica.

CE system equipped with amperometric detection was used to study the oxidation of chlorophenols with ceric acid and the photodegradation of PCP in presence of titanium dioxide. The detection limit for chlorophenols can be significantly improved by immobilizing an oxidase, e.g., glucose oxidase onto the electrode surface since the oxidation products of chlorophenols can recycle the reduced glucose to its original active state in the presence of glucose to provide a non-rate limiting source of electron flow toward the electrode. The detection limit of this method has been reported to be about 1 nM [104].

Phenolic acids which are present in wines and other alcoholic drinks can also be analyzed by CE [105] using a 50 mM hydrogen carbonate buffer at pH 8.3 and an applied potential of 15 kV.

17.5.3 Dyes

In general CE separation is based on the different electrophoretic mobility of ions, and

can be influenced by several parameters, i.e. pH and electrolyte concentration. In the case of dyes, the separation is often difficult to carry over by varying the pH because they may have moieties that are strong acids. Therefore, two methods were applied to adjust the electrophoretic mobility for the separation of six sulfonated azo dyes and other related compounds [106]. Complexation by bis-tris-propane (BTP) and interaction with linear polymers added to the buffer and acting as pseudo-phases were performed. A buffer system based on BTP containing polyethylene glycol and polyvinylpyrrolidone permitted the separation of all analytes. Retention of the dyes caused by the polymeric additives was related to the solute's structure. It was demonstrated that the relative decrease in the electrophoretic mobility of the dyes correlates with the number of benzoaromatic rings in the molecules. Other buffers with different micellar agents like borate at pH 8.3 with cholic acid were employed for the determination of several sulfonated azo dyes and other compounds classes [107]. Analysis of spiked water samples with SPE and recovery studies were also carried over. Burkinshaw et al. [108]used a micellar buffer system consisting on 10 mM $Na_2B_4O_7$–40 mM SDS, pH 9 to separate two sulfonated azo dyes with very similar structure and relative molecular mass, which could not be separated by a conventional buffer, as well as several anionic and cationic dye intermediates. Suzuki and coworkers [109] also used 10 mM SDS as micellar agent in a mixture of 25 mM sodium phosphate buffer and 25 mM sodium borate buffer (1:1) at pH 8.0 for the analysis of sulfonated azo dyes and xanthene dyes used as food additives in Japan. But the isomeric dyes R-2 and R-102 were not separated with good resolution and β-cyclodextrin instead of SDS was added to achieve a good separation. Although most of the authors reported that separation of dyes by free zone capillary electrophoresis is not possible and MEKC has to be applied, in some cases these compounds have been successfully separated by CZE [110].

Quantification studies were also done by Liu et al. [111] using a capillary zone electro-phoresis method for the separation of six synthetic food colorants including four sulfo-nated azo dyes and one arylmethane dye. A linear relationship between the standard concentration and peak area of each of these pigments was obtained in the concentration range of 2–50 ppm, with a correlation coefficient greater than 0.995. The RSD of the method was about 3% and the minimum detectable amount at a signal-to-noise ratio of 3 for all six synthetic colorants was 3 ppm.

Calibration curves for standard solutions of eight sulfonated azo dyes as well as for extracted spiked groundwater samples with the same compounds were also carried out by Schönsee et al. [112]. Additionally, to evaluate the performance of the automated off-line SPE–ASPEC XL–CE/UV method, groundwater samples were spiked with the mixtures of eight dyes in the range of 0.05–0.25 mg/l. The pre-concentration procedure was carried out with the volume of 300 ml in order to achieve the calibration range from 10 to 50 mg/l. The LODs for the different compounds ranged from 11 to 300 ppb. The regression equations were characterized by correlation coefficients higher than 0.95 and better LODs could be calculated for a signal-to-noise ratio of 3 between 10 and 150 μg/l. Fig. 17.16 shows the separation of the target compounds spiked in groundwater (3 mg/l) after off-line solid-phase extraction, that allows the determination of all the dyes.

Detection limits between 11 and 300 ppb for eleven synthetic food colorants (including some sulfonated azo dyes) were achieved using CZE with the host-guest complexation effects of β-cyclodextrin [113]. A 300 μm i.d. capillary tube made of fluorinated ethylene-propylene copolymer in a hydrodynamically closed separation compartment was used for
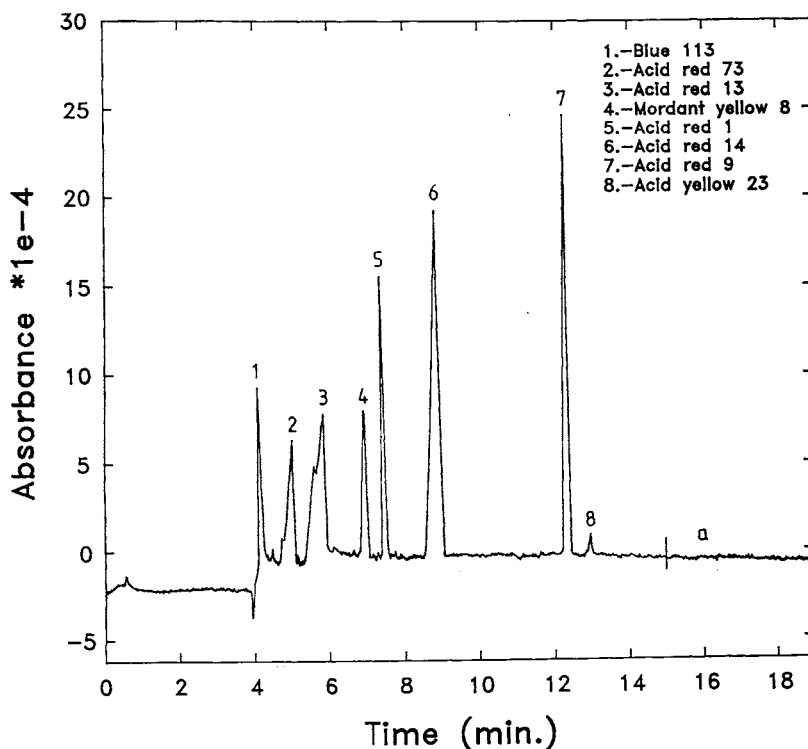
Fig. 17.16. Electropherogram (214 nm) of the separation of eight sulfonated azo dyes spiked in groundwater (3 mg/l) after off-line solid-phase extraction of 300 ml of water with Isolute ENV + cartridges. Separation was carried out with a buffer solution of ammonium acetate 9.2 mM and Brij 35 0.05%. Peaks: (1) Acid Blue 113, (2) Acid Red 73, (3) Acid Red 13, (4) Mordant Yellow 8, (5) Acid Red 1, (6) Acid Red 14, (7) Mordant Red 9 and (8) Acid Yellow 23.

the analysis accommodating 90 nl sample injection volumes, thus providing the above mentioned detection limits. This CZE procedure was applied to several samples including a soft drink concentrate.

Capillary electrophoresis is specially useful to analyze certain reactive dyes that HPLC often has difficulty in analyzing, like the bifunctional β-sulfatoethylsulfone reactive dyes and the phthalocyanine-based dyes. CE was employed in checking the purity of reactive dye samples, monitoring the reactions (kinetic studies) of reactive dyes with nucleophiles or analyzing colored effluents [114]. A variety of buffers were investigated but the use of acetonitrile at a ratio of 1:9 in a micellar buffer system (10 mM SDS, 10 mM sodium tetraborate and 6 mM potassium dihydrogen phosphate at pH 9.0). Detection was by an on-line UV-vis detector positioned at the cathode. A CZE method with UV detection was developed for screening black reactive dyes and black acid dyes, isolated from cotton and wool materials [115]. The dye components were identified by using a newly developed marker technique, using phenylacetic acid, benzoic acid and *meso*-2,3-diphenylsuccinic

acid. With this marker technique the relative standard deviations of the migration indices and the electrophoretic mobilities for the analytes were improved and were below 0.6%.

Other reactive textile dyes like chlorotriazine and vinylsulfone dyes have also been analyzed as well as their hydrolysis products in several commercial dyebath samples and sewage effluents from a water treatment plant [116]. In these samples the majority of the peaks could not be assigned to any known compound and it was concluded that it would be necessary to couple CE with mass spectrometry.

17.5.4 Other compounds

Other compounds like fatty alcohol ethoxylates (FAEs), which are applied in complex formulations (laundry detergents) as mixtures of alkyl and ethoxylate homologues, can also be analyzed by CE. A CE separation method was developed and compared with a well-established HPLC used as reference and supplementary method [117]. UV detection after derivatization with phenyl isocyanate arc light scattering detection has been carried out. The identification of homologues was possible by comparison with peak patterns of substances with known composition or by standard addition. Fig. 17.17 shows the separation of alcohol ethoxylates mixtures by this method. The authors concluded that CE can be used as a supplementary technique and an alternative to HPLC for samples without very complex composition. Moreover in the analysis of these products, where high surfactant concentrations are found, and the higher detection limit of 1 order of magnitude of CE is not a handicap. The reproducibility of peak area was comparable to that of HPLC and the migration times were stable (2% RSD within a day). The advantages of CE (fast, efficient, low solvent consumption, rapid method development) could make this method useful for analysis of FAE in product control.

The practice of chlorination as a means for disinfecting drinking water has been found to be responsible for the production of chlorinated organic compounds [118]. The US Environmental Protection Agency (EPA) has promulgated regulations to control disinfection by-products (DBPs) [119]. Therefore, methods for directly analyzing haloacetic acids without the need for derivatization have been developed. Reversed-phase ion-pair chromatography (RP-IPC) with indirect detection has also been used to determine some of these compounds using UV detection, but the analysis time was long and the detection limits were not very good [120].

For these reasons CZE methods for the determination of haloacetic acids in water have been developed [121]. A CZE system for determining haloacetic acids in water was optimized with indirect photometric detection [116]. Two different electrolytes, potassium hydrogenphthalate and sodium 2,6-naphthalenedicarboxylate, were evaluated in terms of sensitivity and two different electroosmotic flow modifiers were tested. Parameters such electrolyte concentration and pH, and concentration of the electroosmotic flow modifier, which affect the CZE separation were investigated. Of the two electrolytes studied, the latter has better sensitivity and selectivity for these compounds. On the other hand the concentration and type of electroosmotic modifier show no significant effect. The method enables the studied compounds to be determined in 8 min. This shorter analysis time is an advantage over the GC EPA method, where analysis of up to 30 min are normally required. Another advantage is that no derivatization step is necessary to analyze these compounds. Liquid–liquid extraction was used to enrich these compounds from natural waste waters.
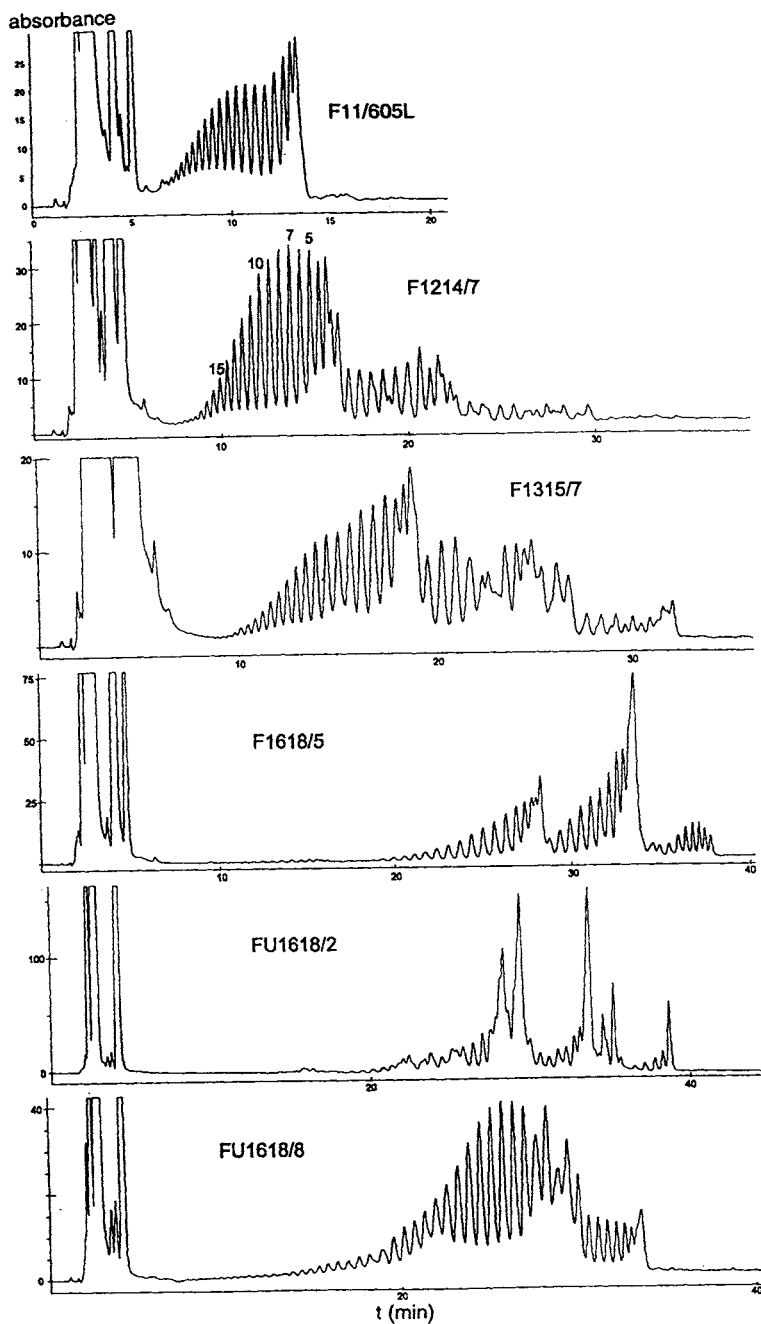
Fig. 17.17. Separation of technical alcohol ethoxylates mixtures (phenyl urethanes) by HPLC with UV detection, column, RP-8 (250 × 4 mm); eluent, acetonitrile/water (70:30) in 40 min to 100:0; detection, UV, 235 nm; flow, 1.5 ml/min; numbers, ethoxylation degree of the homologue; samples, 2.4 g/l F11, 2.8 g/l F1214, 4 g/l F1315, 5.1 g/l F1618 (Präwozell products).

The same authors have revised the alternatives to enhance sensitivity in CE analysis for haloacetic acids, phenols, herbicides and other compounds [73]. Sensitivity improvement both inside and outside the capillary allows the determination of these compounds at low levels (µg/l).

17.6 ENVIRONMENTAL APPLICATIONS OF CE–MS DETECTION

Besides the numerous papers describing CE and CE–MS applications in other areas, e.g. biochemical area, a significant number of reports illustrate the importance of this technique in the field of small molecule and environmental analysis.

A liquid junction CE–CF-FAB–MS system was used to separate a mixture of eight sulfonylurea pesticides [122]. Full-scan collision-induced dissociation spectra of each component was recorded. SIM–MS electropherograms show good efficiencies for most of the peaks and the use of shorter CE columns (length less than 50 cm) allowed shorter analysis times. The need of improvement of the concentration detection limit of the method was emphasized.

Quaternary ammonium salts were used in the initial studies of the CE–ES–MS interface [123], reporting absolute detection limits that ranged from low femtomolar to attomolar. These detection limits could be explained by the high values of the electrospray liquid-gas phase transfer coefficients that are typical of quaternary ammonium cations. Paraquat and diquat can also be analyzed in 7–10 min at pH 3.9 in 50% methanol water using different acetate and formate buffers and its positive ion electrospray mass spectra has been examined [124].

Other groups of pesticides and other contaminants have been analyzed recently, mainly by CE with the ES interface. The determination of chlorinated acid herbicides and several phenols in water was investigated using CE–ES–MS [125]. Sixteen analytes were separated in 40 min with no sample preparation besides pH adjustment with a buffer consisting of 5 mM ammonium acetate in isopropanol-water (40:60 v/v) at pH 10. Quantitative analyses with an internal standard gave relative standard deviations in the range of 3–10%. Improvement of sensitivity with sample concentration techniques, sample stacking and improvements in instrument design indicate that environmentally usefully detection limits are available, as can be seen in Fig. 17.18.

MEKC analysis with MS detection has been achieved by on-line combination of partial filling micellar electrokinetic chromatography (PF-MEKC) and electrospray ionization mass spectrometry [126]. It has been demonstrated for the analysis of triazine herbicides, that PF-MEKC involves filling a small portion of capillary with SDS micellar solution for achieving the separation. The triazine analytes first migrate into the micellar plug where the separation occurs and then into the electrophoresis buffer which is free of surfactant. Consequently, the electroosmotic transfer of neutral triazine herbicides to ES–MS is comparable to conventional CZE–MS. Therefore PF–MEKC–ES–MS provides a mechanism for the separation and mass detection of neutral molecules without the interference of surfactant.

A laboratory-made CE system with relatively short (25–35 cm) capillaries has been coupled to a pneumatically assisted electrospray interface for separations of sulfonylureas with on-line MS detection [127]. It is not straightforward to implement the use of short
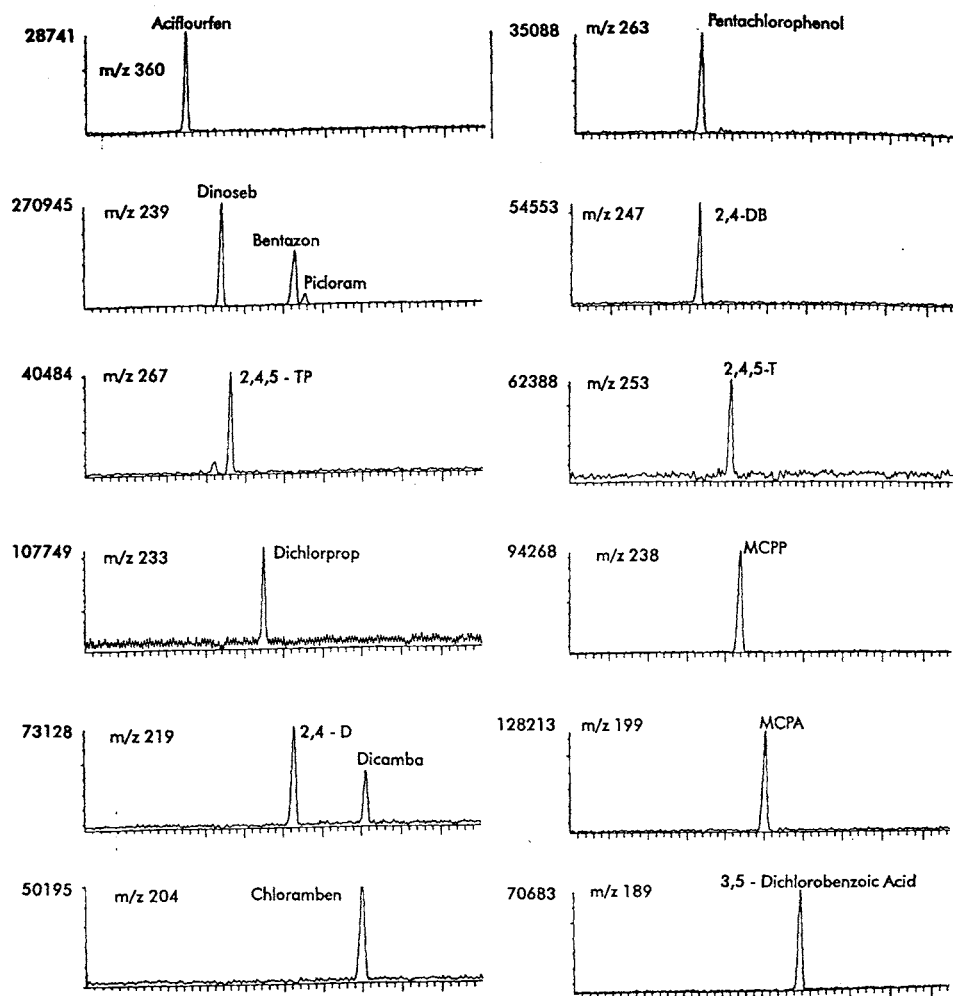
Fig. 17.18. Selected ion electropherograms from the CE separation of nine of the analytes at the concentration of 50 μg/l and the internal standard at 250 μg/l with sample stacking on the CE column.

capillaries (less than 50 cm) for CE–MS with many commercial CE instruments because the relatively large size and configuration of these instruments. The use of high-voltage potentials (800–1000 V/cm) created considerable Joule heating and sensitivity should be improved in future works. In summary, the laboratory-made system described in this work does not have the versatility of commercial units but was a good alternative approximation for the routine analysis of the studied compounds.

CZE has also been coupled to MS on-line via a tricoaxial sheath flow interface with a benchtop quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ionization source [128]. This system has been applied to the analysis of the fingerprinting of an alkylsulfate detergent and to the impurity in a chloroamine-T disinfectant,

the analysis of phenoxyacid herbicides, the characterization of a complex polyethoxylated alkylphosphate emulgator and related impurities. The sensitivity, typically in the picogram range under full-scan conditions, enabled the identification of unknown minor impurities. ITP–CZE–MS under high electroosmotic flow conditions, yielding extremely narrow peaks (up to 4 million theoretical plates), was found to be very useful for on-column preconcentration and subsequent identification of trace impurities [123].

A comparative work of capillary liquid chromatography (cLC) and CE on the separation of some sulfonated and other classes of dyes has been carried out [129]. MEKC provided excellent separation and quantification of synthetic dyes and was found to be complementary to the reversed phase of cLC in the separation and in their ability to quantify and identify analytes. Recovery data for water and soil extraction were obtained by MEKC–UV detection. Although cLC was coupled with continuous-flow liquid secondary ion mass spectrometry (CF-LSI–MS) for confirmation purposes, interfacing CE to the MS using a coaxial arrangement as used in cLC was more difficult and only UV detection with CE was performed.

Ion evaporation appears to be the preferred ionization mechanism for on-line CZE–MS because separated components already exist as charged species in the CZE buffer. Furthermore this ionization process is best-suited for species which exist as ions in solution such as sulfonated azo dyes.

CF-FAB has been coupled to CE [130] with a coaxial interface to analyze aromatic sulfonic acids and some azo dyes. However, excessive band broadening and loss of
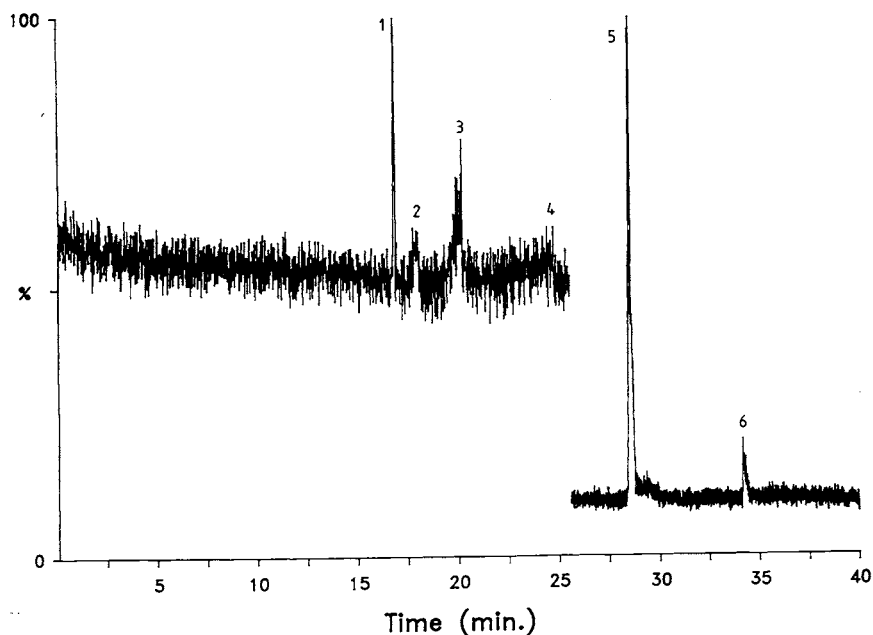
Fig. 17.19. TIC of a CE–MS electropherogram for an extracted spiked (3 ppm) water sample. Peaks: (1) Internal Standard, (2) Acid Red 73, (3) Acid Red 1 + Mordant Yellow 8, (4) Acid Red 13, (5) Acid Red 14, (6) Mordant Red 9.
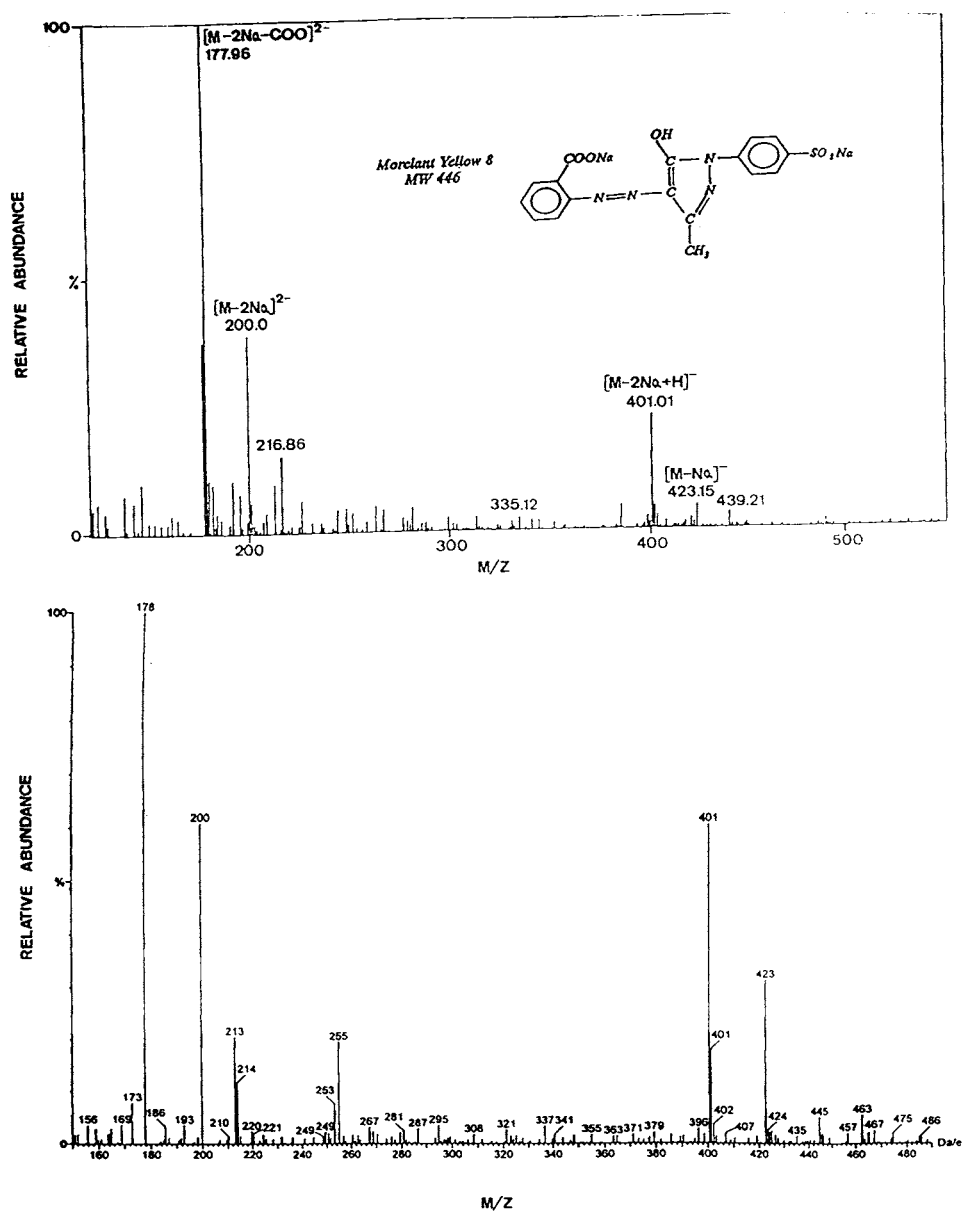
Fig. 17.20. Mass spectra of Mordant Yellow 8 with (A) LC–ISP–MS interface and (B) CZE–MS interface.

separation efficiency occurred owing to the long transfer line to the FAB ion source and the high vacuum requirements. The newly developed atmospheric pressure interfaces like ionspray produce a mild form of ionization described by Iribarne and Thomson [131]; it was first coupled to CE by Henion and co-workers [132,133] and it was applied by Riu
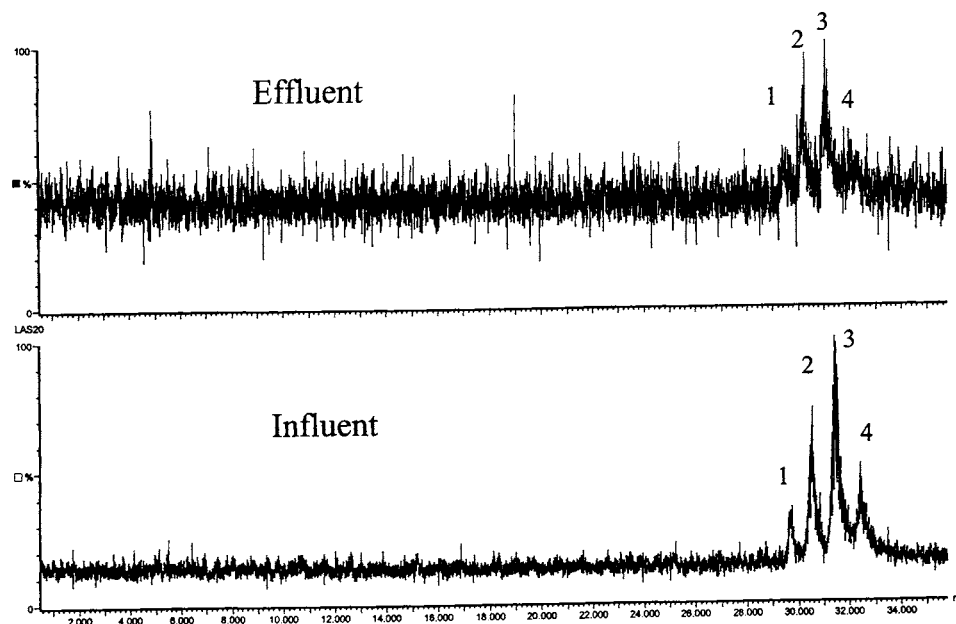
Fig. 17.21. Selected ion electropherograms from the CE separation of the LAS in a waste water plant effluent and influent. Peaks: (1) C10 LAS; (2) C11 LAS; (3) C12 LAS; (4) C13 LAS.

[129] for determining sulfonated azo dyes. Detection limits range from high parts per billion to low parts per million. The correlation coefficient for the linear regression through the means of four levels of concentration of the dye injected was found to be 0.990 and the reproducibility for automatic injection could routinely be within 2–4%. The mass spectra of the azo dyes gave peaks $[M-nH]^{n-}$, depending on the number of sulfonic groups. CE tandem mass spectra of the deprotonated molecular parent ion showed a daughter ion at m/z 80 corresponding to the sulfonated ion. Three sulfonated azo dyes were detected at low parts per million in spiked wastewater extract monitoring the sulfonic ion at m/z 80.

Eight mono- and disulfonated azo dyes have been recently analyzed with a Beckman P/ACE capillary electrophoresis system connected with a Micromass interface to a VG Platform MS instrument [134]. Spiked and extracted water samples were separated and determined for confirmation of the studied compounds and a typical chromatogram of the analysis of an extracted sample is shown in Fig. 17.19. Water samples were analyzed at a 3 ppm level by monitoring one ion for each studied compound in order to achieve maximum sensitivity. Detection limits between 100 and 800 ppb were achieved with the exception of Acid Red 73 that exhibited low response and could not be quantified. Compounds which coeluted and were not resolved could be determined because ions with different m/z ratio were obtained. The spectra obtained by CE–MS were similar to those obtained with ISP ($[M - t\mathrm{Na} + (t + z)\mathrm{H}]$ ions, depending on the number of sulfonated groups (n) and the charge (z), and some losses of SO_3^- and COO^- groups). In a previous work from the same

laboratory [135], the spectra of one of the studied dyes are compared using both interfaces (Fig. 17.20).

A method for the determination of anionic surfactants (linear alkylbenzenesulfonates, LAS) in the influent and affluent of waste water treatment plants has also been developed by CE with UV detection and confirmation was achieved by CE/MS detection [136]. In Fig. 17.21, a selected ion chromatogram of a CE separation of the LAS in a waste water effluent real sample, is shown. The LODs were higher than those obtained with other techniques like LC with fluorescence or MS detection, and were in the range of 10–100 ppb when combined with a preconcentration method (i.e. SPE). For this reason this technique is only amenable to the analysis of samples with high concentrations of surfactants as in fact are the waste water treatment plant effluents.

17.7 CONCLUSIONS AND FUTURE DEVELOPMENTS

Capillary electrophoresis involves a group of separation modes that can provide high resolution separations in short analysis times. Instruments have become commercially available and are similar in price to LC systems. Few manufacturers build instruments with good quantitative injection precision and accuracy (without the need for an internal standard), thermostated capillary compartments, and variable wavelength UV absorbance detectors with excellent signal-to-noise characteristics. Compared to chromatographic separation systems, CE is more economical; with no expensive columns, only small volumes of buffer solutions, less maintenance and less method-development effort.

CE is very suited for those analytes that are not amenable to GC, when existing LC methods do not offer sufficient separation power or for ionic compounds. Many impressive CE separations, including the separation of pollutants, have been demonstrated in the last few years. Due to the recently developed focusing injection techniques, CE has become competitive in trace analysis and the door has been opened to environmental applications in real matrices. But still the lack of sensitivity is a problem for most routine environmental applications.

It can be predicted that a wider range of CE detectors will be offered by the instrument manufacturers. Current UV absorbance detectors were not specifically designed for CE, but are modified LC detectors. These detectors might be replaced by sophisticated detectors based on fibre-optic technology. More laser-based fluorescence detectors will be offered, and the high stability inexpensive diode lasers (with frequency doubling) might be incorporated in CE instruments at a later stage. By using very selective and sensitive detectors like fluorescence, it has been possible to achieve LODs below 0.1 ppb with only 200 ml of water extracted [137]. Conductivity detection and electrochemical detection will be commercialized as well. One of the most interesting instrumental development, however, has been the introduction of a bench-top CE–MS with an atmospheric pressure electrospray/ion-spray source. Capillary electrophoresis can be easily coupled with classical ESP interfaces because of the restricted flow rates. Sensitivity problems result from the small injection volumes and pre-concentration techniques should be applied. More work will be done in the near future towards the application of CE–MS combined with solid-phase extraction. The high efficiency and diversity in chemistry separation of CE as well as the fact that is an inexpensive and low generator of waste technique will permit to

achieve the required analytical performance for trace determination of a wide range of pollutants in environmental matrices.

It is to be expected that CE instruments will be introduced in more and more analytical laboratories and the number of applications will continue to grow. The nature of these papers have been changing gradually from 'just showing a particular separation', towards applications in real environmental matrices. However, despite the excellent prospects for CE, many potential CE users may hesitate for the next few years, simply because the official methods currently used in environmental trace analysis do not incorporate relatively new techniques, like CE. It is also expected that new developments like CEC-MS will be possible in the near future and will permit solutions for analytical problems in the area of environmental chemistry.

ACKNOWLEDGEMENTS

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Chapter 18

Application of fluorescence spectroscopic techniques in the determination of PAHs and PAH metabolites

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CONTENTS

18.1 INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are usually defined as a group of chemicals consisting of two or more fused benzenoid rings and containing no other elements than hydrogen and carbon. In general, they are highly fluorescent, and fluorescence spectroscopic techniques are frequently used for the analysis of these compounds and their derivatives.

PAHs are natural constituents of crude oil and many other petrochemical products. These aromatic structures have been formed during the course of millions of years by

successive dehydrogenation of organic material at relatively low temperatures. In PAH mixtures of petrochemical origin two- or three-ring compounds, such as naphthalene, phenanthrene, and their alkylated derivatives, are more abundant than the heavier PAHs containing four or more rings. PAHs can also be formed at more elevated temperatures during the inefficient combustion of fossil fuels or other organic matter. A typical PAH mixture of a high-temperature combustion source contains mainly unsubstituted compounds, and the PAHs of four and more rings are more abundant than the smaller ones. PAHs in natural samples are always encountered as mixtures: the relative distribution of the various PAH congeners, the PAH 'profile', provides a clue to the origin (petrochemical or pyrolytic) of the pollution. Natural sources of PAH emission have always existed (sediment erosion, volcanic eruptions, forest fires), but since the industrial revolution there has been a rapid increase in the loading of the environment with petrochemical PAHs (oil spills) and particularly with PAHs of pyrolytic origin.

Many PAHs are proven or suspected carcinogens, the most well-known example being the 5-ring aromatic compound benzo[*a*]pyrene, BaP. It should be realized, however, that BaP itself is relatively inert; it needs to be metabolized before it can exert genotoxic activity. Biotransformation often starts with the binding of the xenobiotic compounds to the cytochrome P450 enzyme system, which catalyzes the addition of an oxygen atom across a double bond of the molecule, thus forming an epoxide. This epoxide may subsequently be coupled to glutathione, isomerize into a phenol, or be hydrolyzed to yield a saturated dihydrodiol moiety. Phenols and dihydrodiols can be conjugated to glucuronic acid or sulfate to facilitate excretion. Overall, the biotransformation of the strongly lipophilic BaP results in the formation of more polar metabolites that are more easily excreted than the parent compound. The result of these detoxification mechanisms is that the bulk of PAH molecules, after absorption by higher species, is also rapidly removed from the body. However, some reactive intermediates formed during the process may form adducts with proteins or with DNA. The latter could lead to the initiation of cancer if the defective nucleotide is not repaired in time. Other mechanisms, such as enzyme-catalyzed one-electron oxidation, have also been reported.

The challenge for analytical chemists is not only to develop methods for the identification and quantitation of PAHs in various environmental samples; also the amount of a specific PAH that is actually absorbed by a given organism needs to be determined. In this chapter the attention is focused on the development and application of fluorescence techniques for this purpose. The two spectroscopic methods described can be applied to whole samples without the need for chromatographic separation. Since conventional fluorescence spectroscopy is not appropriate for the analysis of mixtures of PAHs in real samples, enhancement of selectivity has to be realized. Two approaches are followed, i.e. Shpol'skii spectroscopy, a technique providing highly specific fluorescence spectra (with fingerprinting characteristics) for certain analytes in (poly)crystalline matrices at cryogenic temperatures, and synchronous fluorescence spectroscopy (SFS), a conventional, room temperature technique based on the synchronous scanning of excitation- and emission wavelengths.

Extensive attention will be paid to applications of Shpol'skii spectroscopy and SFS to sediment and biota samples for environmental analysis. The Shpol'skii method will be used for the determination of parent PAHs, including the extremely potent carcinogen dibenzo[*a,l*]pyrene. Also important is its application to the identification and quantitation

of PAH metabolites in bile of fish exposed to sediments with different PAH pollution levels. Thus, a direct indication of the amounts entering the body can be obtained. Laser excitation was used to increase both the selectivity and the sensitivity of the method. SFS is not sensitive enough to detect BaP metabolites in fish bile. Nevertheless, a correlation was found between 3-hydroxy-BaP and 1-hydroxypyrene concentrations, and the latter can be readily determined with SFS. Although pyrene is not believed to be particularly toxic, the determination of its major metabolite 1-hydroxypyrene in bile samples by means of SFS would be a suitable screening method for the biomonitoring of total PAH exposure, provided the PAH uptake profile is more or less constant.

Though this chapter is mainly concerned with parent PAHs and their hydroxy-substituted metabolites, some attention will also be paid to recent Shpol'skii experiments on AT-ring and IN-ring hetero-substituted PAHs, in particular to amino- and nitro-PAHs and to azaarenes (i.e. in-ring nitrogen-substituted PAHs). These types of compounds are known to be present, in addition to PAHs, in various environmental matrices of both natural and anthropogenic origin. Since they induce mutagenic and carcinogenic activity, the analytical interest is self-evident. Furthermore, their correct qualitative and quantitative determination is needed to advance our understanding of the environmental fates of PAHs. Nitro-PAHs are for instance formed from parent PAHs through reaction with nitric acid and nitrogen oxides, while, under anaerobic conditions, chemical reduction may lead to conversion of nitro-PAHs to amino-PAHs.

18.2 FLUORESCENCE SPECTROSCOPY

When solid or fluid solutions are irradiated with ultraviolet or visible light, sometimes luminescence (fluorescence and/or phosphorescence) is observed, i.e. the sample emits light of longer wavelength than the excitation light in all directions. For a solute molecule the energy diagram, usually denoted the Jablonski diagram, and the relevant transitions and decay processes are depicted in Fig. 18.1. Through the absorption of light, molecules (or atoms) may be promoted to a higher electronic state (S_1, S_2, etc.). The energies and probabilities of these transitions can be studied by recording an (electronic) absorption spectrum. In the condensed phase, relaxation of the excited molecule to the lowest vibrational level of the S_1 state (via internal conversion and vibrational relaxation), is usually very fast. The remaining excitation energy stored in the molecule may also be lost nonradiatively, or may be transferred intermolecularly to other chromophoric groups in the sample or intramolecularly within the same molecule. For a limited number of compounds, however, the return from S_1 to the electronic ground state S_0 is accompanied by the emission of a photon (fluorescence). In some cases, phosphorescence from the lowest vibrational level of the triplet state T_1 takes place. Fluorescence and phosphorescence spectra yield information on the vibrational levels of the electronic ground state S_0, and are as such complementary to the absorption spectrum.

Luminescence spectroscopic techniques are widely recognized as useful tools in analytical chemistry, especially because of their high inherent sensitivity (zero-background measurement). Obviously, the fact that only a restricted group of analytes displays fluorescence implies that the method is on the one hand not universally applicable, but at the same time more selective. Polycyclic aromatic hydrocarbons usually show strong fluor-
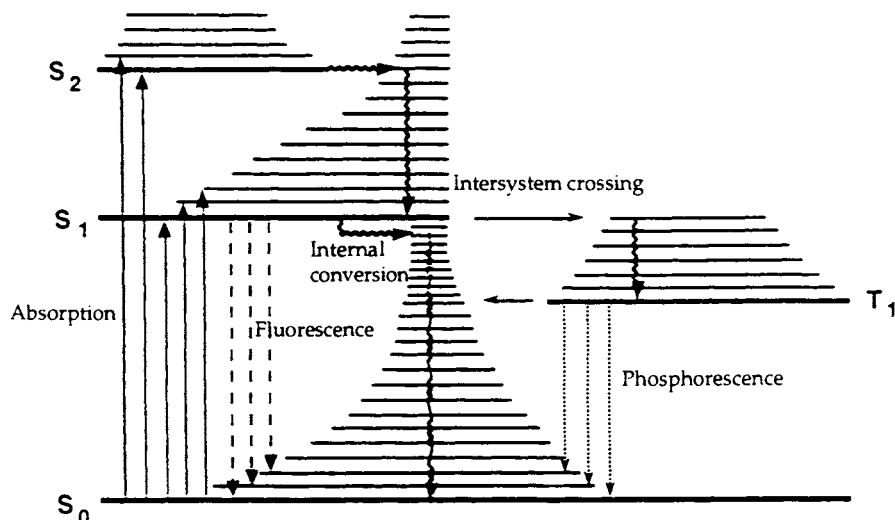
Fig. 18.1. Jablonski diagram of molecular system, showing relevant electronic transitions and decay processes. S, singlet state; T, triplet state. Reproduced from [2], with permission.

escence, and several fluorescence-based techniques have been developed for PAH analysis. For the determination of mixtures of PAHs, for instance in petrochemical or environmental samples, fluorimetric detection usually takes place after some form of (chromatographic) separation. Separation is necessary, as (rather unexpected in view of the discrete levels shown in Fig. 18.1) the fluorescence spectra of molecules often consist of rather broad bands that provide little information on the identity of the compound. Furthermore, fluorescence spectra of different compounds often show severe overlap. Identification and proper quantitation of analytes in a mixture is not possible under conventional conditions.

A simple method that leads to some spectral simplification and reduced overlap is known as synchronous fluorescence spectrometry (SFS). A more significant increase in spectral resolution may be obtained by means of several low-temperature techniques: fluorescence line-narrowing spectroscopy, supersonic jet spectroscopy, matrix isolation spectroscopy, or Shpol'skii spectroscopy (SS). For the analysis of PAHs and PAH metabolites the Shpol'skii technique proved particularly useful, as demonstrated below.

18.2.1 Shpol'skii spectroscopy

In order to understand the Shpol'skii effect the question should be answered why, for molecules in the condensed liquid or solid phase, broad-banded spectra are observed instead of discrete, sharp lines. Irrespective of other mechanisms, the main cause is inhomogeneous line broadening: each individual analyte molecule experiences a different influence of its surrounding solvent cage. Thus, each molecule has its own Jablonski diagram and the energies of the electronic states are slightly different. As a result, a Gaussian distribution of narrow lines is observed with a total band width of typically several hundred cm^{-1} for each transition.

In Shpol'skii spectroscopy, the inhomogeneous broadening induced by the matrix is largely diminished. Shpol'skii and coworkers [1] observed a dramatic line-narrowing effect in the fluorescence spectrum of coronene on rapid cooling to 77 K in *n*-hexane or *n*-heptane solutions. The use of organic solvents that form amorphous, glassy matrices at low temperatures did not produce high-resolution spectra. The phenomenon, which soon became known as the Shpol'skii effect, is illustrated in Fig. 18.2a,b, which depicts the fluorescence spectra of benzo[*k*]fluoranthene in *n*-octane at room temperature and at 26 K, using the same experimental setup [2]. Since the total fluorescence intensity is at least equal and often even higher at low temperatures, the line-narrowing effect results at the same time in an increase in signal height of two orders of magnitude. The sensitivity of the Shpol'skii technique will be discussed in more detail in the section on analytical applications.

It is generally believed that the narrow-banded or quasilinear emission lines are produced by isolated molecules, trapped in the matrix during the cooling procedure and substituting one or a few solvent molecules in the (poly)crystalline lattice [3–5]. When the analyte can occupy two or more different sites in the matrix, a multiplet spectrum will be observed, as the individual spectra of different subsets of molecules are shifted with respect to each other as the result of different matrix interactions (different 0–0 energies). The Shpol'skii spectrum of 1-hydroxy-benzo[*a*]pyrene in *n*-octane (Fig. 18.3) is an example of a doublet spectrum [2]. The intensity ratio of the various emission lines within a multiplet is the same for each transition and reflects the distribution of the analyte molecules over the various sites. When a narrow-banded excitation source (e.g. a dye laser) is tuned to a narrow-banded absorption transition of one of the analyte subsets, a single-site spectrum will be observed, as was first demonstrated by Vo-Dinh and Wild [6].

The spectral band widths that can be observed in Shpol'skii systems at temperatures of 20 K and lower are typically 2–10 cm^{-1} (ca. 0.1 nm). Abram and coworkers [7] showed that the line width of a vibronic emission band of perylene in *n*-octane at 4.2 K could be
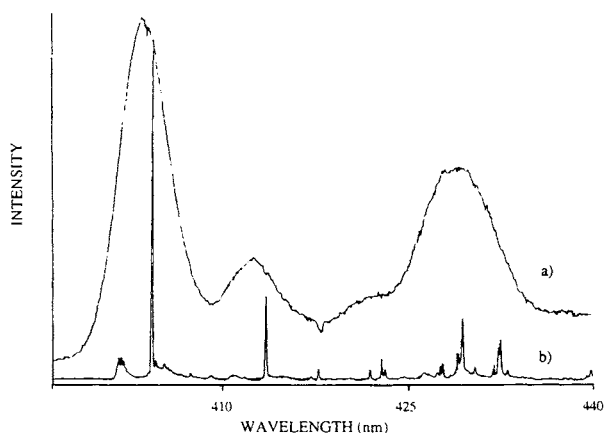
Fig. 18.2. Emission spectra of benzo[*k*]fluoranthene in *n*-octane, using xenon lamp excitation at 308 nm. (a) Room temperature spectrum, concentration $= 10^{-4}$ M. (b) Shpol'skii spectrum at 26 K, concentration $= 10^{-6}$ M. Spectra were recorded using the same experimental settings; intensities are on the same scale. Reproduced from [2], with permission.
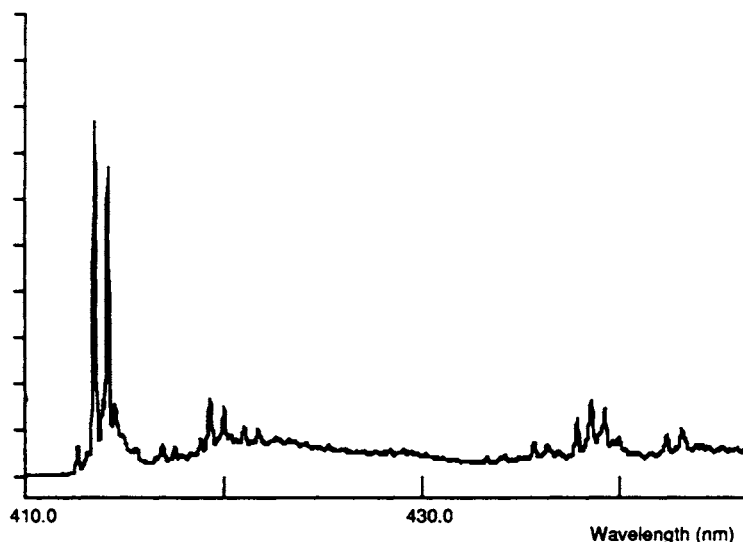
Fig. 18.3. Double-site spectrum of 1-hydroxy-benzo[a]pyrene in n-octane at 10 K; concentration 5×10^{-6} M; xenon lamp excitation 295 nm. Reproduced from [2], with permission.

reduced from 4 cm^{-1} to the instrumental limit of 0.4 cm^{-1} by selective excitation with a laser. This illustrates that some inhomogeneous broadening is still present in Shpol'skii systems. In practice, however, the line-narrowing induced by the matrix alone (in a lamp excited experiment) is often sufficient for isomer-specific identification and for the determination of complex mixtures.

Whether a given analyte will produce Shpol'skii-like emission in a particular matrix depends on the compatibility of the physical and geometrical properties of the host–guest combination. For instance, naphthalene 'fits' in an n-pentane crystal, but yields only broad-banded emission in n-hexane and n-heptane. This phenomenon is usually referred to as the 'key and hole principle', although it appears that the geometric requirements are less stringent for most larger PAHs containing four or more rings, as they produce good Shpol'skii spectra in a range of n-alkane matrices [4,8].

Schematically, an instrumental setup for Shpol'skii fluorescence measurements consists of the following modular components: (1) excitation source, often with some type of wavelength selector; (2) low-temperature sample holder; (3) high-resolution emission monochromator; (4) detector. At present, no complete turn-key instruments are available on the market, but a Shpol'skii setup can be assembled from commercially available components.

18.2.1.1 Excitation sources

The Shpol'skii effect is a matrix-induced phenomenon; the use of highly monochromatic (laser) excitation can be advantageous, but is not a prerequisite for observing narrow-banded spectra. Frequently mercury or xenon arc lamps in combination with an excitation monochromator are applied. Laser sources are utilized to improve selectivity

and/or sensitivity. For PAHs it is known that also the S_1–S_0 part of the absorption spectrum consists of narrow lines. Selective excitation is possible if the laser wavelength can be tuned to a specific absorption line of the particular analyte under investigation. Nowadays, several types of lasers and dye lasers are available which -in combination with frequency doubling, frequency tripling, frequency sum mixing or Raman shifting techniques- cover a wide range of relevant wavelengths. For the laser-excited Shpol'skii experiments described below, an Nd:YAG laser (frequency doubled) was used in combination with a dye laser. For the measurements of benzo[a]pyrene metabolites the Nd:YAG setup could be easily switched from frequency mixing of the dye laser output (for selective excitation around 420 nm) to frequency doubling (for nonselective excitation around 350 nm). Alternatively, a XeCl excimer laser–dye laser combination could be used.

18.2.1.2 Cryostats

Although the Shpol'skii effect was first observed at a temperature of 77 K, the boiling point of liquid nitrogen, the use of lower temperatures results in a substantially better spectral resolution [2]. Various types of helium cryostats are available; the closed-cycle systems have become very popular because of their ease and low cost of operation (no helium consumption). In these cryostats the circulating medium helium is expanded in the cold station and cools the sample through thermal conductance. Depending of the number of cold stages of the instrument, temperatures of typically 10 or 20 K can be reached. The cooling procedure of the sample obviously affects the freezing rate of the sample, and may thus have an influence on the shape of the spectrum for certain analytes that are not fully compatible with the matrix. Which method results in the most efficient trapping of isolated molecules in crystalline sites will also depend on the sample holder design [9]. Alternatively, helium bath cryostats could be used for experiments at 4.2 K or when a high sample throughput is important (instantaneous cooling).

18.2.1.3 Emission monochromators

Evidently, a good monochromator of moderate or high resolution is required to observe the quasilinear emission spectrum. The spectral resolution of the instrument should preferably be better than 0.2 nm. For trace analysis, the instrument should also have a high light throughput. Holographic gratings, optimized for the wavelength area of interest, are preferred. Often the F/n number of the emission monochromator will be fairly high, which means that some care should be taken with proper focusing of the fluorescence light on the entrance slit.

If nonselective, short-wavelength excitation is applied, scattered excitation light is easily rejected with an appropriate cut-off filter. If, on the other hand, the excitation wavelength is close to the emission lines of interest, as in the case of selective laser excitation in the S_1–S_0 absorption region, the cutoff functions of such filters are usually not sufficiently steep. In that case, the use of a double or even triple monochromator will be very advantageous. For the laser-excited Shpol'skii spectroscopy (LESS) measurements described below [2], a Spex 1877 triple monochromator was used.

18.2.1.4 Detectors

Until about 10 years ago, commonly photomultiplier tubes (PMTs) in combination with a scanning monochromator were used for detection. However, they have the obvious disadvantage that the time required to record a complete high-resolution spectrum is long (typically 10–30 min).

More recently, multichannel detectors have become available that are particularly useful in high-resolution spectroscopy. These detectors are mounted in the (exit) focal plane of the monochromator after removal of the exit slit. The monochromator is thus turned into a spectrograph; measurements are usually carried out at a fixed wavelength position, although operating the detector in the scanning mode can have certain advantages [10]. The intensified linear diode array (ILDA) detector consists of typically 512 or 1024 separate photodiodes (center to center distance ca. 25 μm), that release electrons on the absorption of light. At the end of an integration cycle defined by the operator, the accumulated charge in each photodiode-capacitor pair is sampled, digitized, and fed into a computer. Since the read-out of such detectors is accompanied by considerable electronic noise, the use of an intensifier (a combination of a photocathode, a multichannel plate, and a phosphor screen, which multiplies the number of incident photons with a gain of typically 10^3) is indispensable for the detection of low light levels. Fast gating of the ILDA detector is possible by means of a fast pulser unit that switches the photocathode of the intensifier unit on and off at specific times.

Another type of multichannel detector is the charge-coupled device (CCD) detector. These detector chips consist of a plane of semiconductor material and a fine electronic network that divides the chip into a matrix of small squares (pixels). Typical CCD chips presently contain 512×512 pixels of approximately 20×20 μm each. As the read-out noise of CCD chips is rather low, CCD detectors do not necessarily require an intensifier. With CCD detectors two-dimensional spectra can be recorded [11], but in case of ordinary one-dimensional spectroscopic measurements the charge accumulated in each column can be summed ('binned') for extra sensitivity. A scintillator dye can be applied to the CCD chip for extension into the UV region.

In our experience ILDA and CCD detectors offer roughly comparable sensitivities, when applied to Shpol'skii analysis [2]. In the experiments described below an ILDA was used. When compared to PMT detection, it should be noted that not only the gain in analysis time (ca. 2 orders of magnitude) offered by the multichannel detector plays a role, also the time gating to reject stray excitation light is better in the case or intensified multichannel detectors. An even more important advantage of the multichannel detector is that each data point of the complete spectrum is equally affected by light source instability (slow drift, flicker noise) or by photochemical decomposition of the analyte. These factors will be particularly important when lasers are used for excitation.

18.2.2 Synchronous fluorescence spectroscopy

In conventional molecular fluorescence spectroscopy, two types of spectra are generally discerned: emission and excitation spectra. In order to record an emission spectrum, the compound is excited at a fixed wavelength, while the fluorescence intensity is measured as a function of the emission wavelength. At ambient or lower temperatures, fluorescence

emission of virtually all PAHs takes place after relaxation of the excited molecule to the lowest vibrational state of the first excited singlet state S_1 (see the Jablonski diagram, Fig. 18.1). Thus, the shape of the emission spectrum is independent of the excitation wavelength and only reflects the S_1–S_0 energy difference, the vibrational levels of the S_0 ground state, and the respective transition probabilities. The excitation wavelength thus affects only the absolute intensity of the emission spectrum.

Alternatively, an excitation spectrum can be recorded by measuring the fluorescence intensity at a fixed wavelength, while scanning the wavelength of the excitation light. The absolute intensity of the excitation spectrum may be influenced by the choice of the wavelength monitored, but the shape of the excitation spectrum is emission-independent and only reflects the transitions and transition probabilities from the lowest vibrational level of the S_0 electronic ground state to vibrational levels of excited singlet states (S_1 and higher).

If the excitation wavelength λ_{ex} and the emission wavelength λ_{em} are both varied, the total luminescence function is obtained, which is a multiplication of E_{ex} (a function describing the emission spectrum) and E_{em} (a function describing the excitation spectrum). The total luminescence constitutes a three-dimensional hypersurface, that can be visualized as a pseudo-three-dimensional stack plot or, alternatively, as a contour plot projected onto the λ_{ex}–λ_{em} plane, in which contour lines connect points of equal intensity I (Fig. 18.4). A vertical cross-section through the TL plot produces the conventional excitation spectrum (λ_{em} = constant), while a horizontal cross-section parallel to the λ_{em} axis yields the conventional emission spectrum.

We shall now use the total luminescence plot of Fig. 18.4 to visualize the most impor-
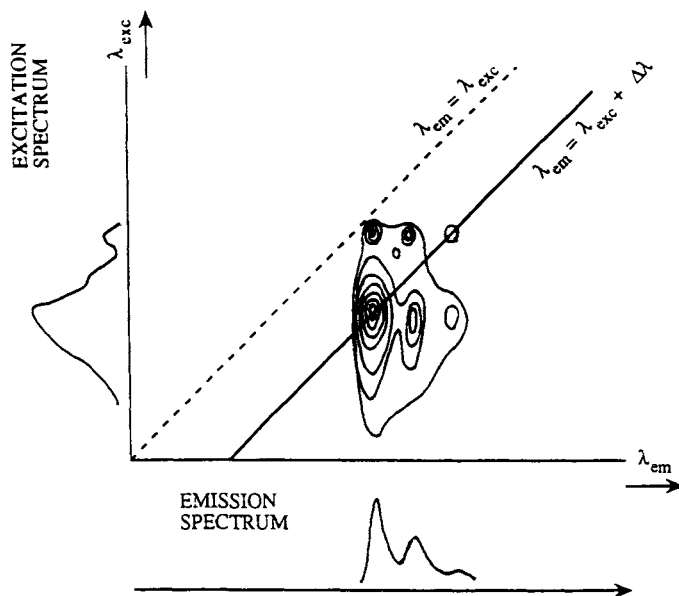
Fig. 18.4. Contour plot of total excitation-emission function of compound showing typical PAH vibrational structure. The synchronous spectrum is represented by the diagonal cross-section $\lambda_{em} = \lambda_{ex} + \Delta\lambda$; Rayleigh scattering occurs at $\lambda_{em} = \lambda_{ex}$. Reproduced from [2], with permission.

tant advantages of the synchronous scanning technique. One can easily imagine that different cross-sections through the TL surface can be obtained if λ_{ex} and λ_{em} are both allowed to vary during the experiment. Although modern spectrofluorimeters with software-driven, independent monochromators could offer an unlimited number of scanning combinations, the traditional approach, as described first by Lloyd [12,13], involved a spectrofluorimeter in which the excitation and emission monochromators were mechanically interlocked, such that $\lambda_{ex} - \lambda_{em} = $ constant. The fluorescence intensity is recorded as the excitation wavelength trails the plotted emission. Lloyd called the spectra obtained this way 'synchronously excited fluorescence emission spectra', although they could also be regarded as excitation spectra with synchronously recorded emission [14]. In Fig. 18.4, the SFS spectrum is represented by a diagonal cross-section through the TL surface, along the line $\lambda_{em} - \lambda_{ex} = \Delta\lambda = $ constant:

$$I_{SFS}(\lambda_{ex}) = Kcl\phi E_{ex}(\lambda_{ex})E_{em}(\lambda_{ex} + \Delta\lambda)$$

If the selected value for $\Delta\lambda$ is rather small, the SFS line slices through a corner of the TL plot, which results in an SFS spectrum that covers no more than a few nanometers. Fluorescence spectra can sometimes be reduced to a single narrow band if $\Delta\lambda$ is chosen to match the Stokes' shift of the analyte in the particular solvent (for PAHs typically 3–6 nm [14]). The wavelength offset $\Delta\lambda$ can not be chosen too small because of Rayleigh scattering at $\lambda_{em} = \lambda_{ex}$. Obviously, spectral reduction leads to loss of information, but this is at the same time one of the main advantages of the SFS method. Reduction of spectral overlap allows the determination of individual PAHs in complex samples, as was demonstrated by Vo-Dinh and Martinez [15].

Especially if the 0–0 band of the excitation and/or emission spectrum is not very intense (e.g. pyrene, BaP), one may prefer to select a larger $\Delta\lambda$ and thus obtain a better sensitivity [15]. In that case, the resulting SFS spectrum will stretch out over a spectral range of approximately $\Delta\lambda$, but may still be much simpler than the conventional fluorescence spectrum. In a conventional emission measurement, the complete spectrum is recorded using the same optimal excitation wavelength. In an SFS measurement, when the wavelength offset is chosen to match the difference between the maxima of excitation and emission, only the emission maximum will be recorded under optimal excitation conditions and with optimal sensitivity. The rest of the spectrum may not be completely removed, but will at least be less efficiently excited. Fig. 18.5 presents the conventional and SFS spectra of the PAH metabolite pyrene-1-glucuronide, using $\Delta\lambda = 37$ nm. The absolute intensity of the SFS peak is equal to that of the conventional emission maximum (same excitation and emission maxima, same slit widths), but the spectrum is confined to approximately 37 nm and the intensity of the vibronic emission bands is strongly reduced.

18.3 APPLICATIONS

18.3.1 Shpol'skii spectrofluorimetric analysis of parent PAHs

In this section it will be shown that SS is an appropriate analytical technique for the determination of PAHs in environmental samples. First of all, SS was invoked as an independent identification technique to upgrade routine HPLC analysis of marine sediment samples. Secondly, it was applied independently to the analysis of PAHs in sediment
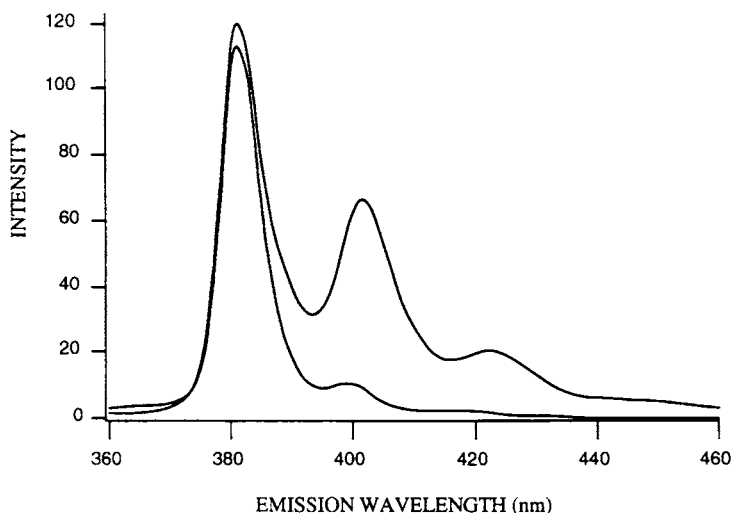
Fig. 18.5. Conventional (top; $\lambda_{ex} = 345$ nm) and synchronous (bottom; $\Delta\lambda = 37$ nm) fluorescence spectra of 5×10^{-8} M pyrene-1-glucuronide in ethanol/water (50:50); intensities are plotted on the same scale; spectral band passes were 5 nm in both experiments. Reproduced from [2], with permission.

reference materials as a quantitative method and compared with existing techniques i.e. LC with fluorescence detection and GC-MS. Laser-excited Shpol'skii spectroscopy was used to determine dibenzo[*a,l*]pyrene, the most potent PAH carcinogen known to date, in sediment samples without chromatographic separation. Finally, the applicability of SS to the analysis of such complex samples as biota containing a large amount of interfering substances (e.g. fatty components) is demonstrated.

18.3.1.1 Identification of PAHs in LC fractions

Gradient LC combined with fluorescence detection is routinely used in the Dutch Water Quality Survey to determine the PAH contents of marine sediment and suspended matter samples. Fig. 18.6 shows a typical chromatogram utilizing a gradient elution program starting with water/methanol 30:70 and ending at 100% methanol. Benzo[*b*]chrysene was added as an internal standard as recommended by the Marine Chemistry Working Group of the International Council for the Exploration of the Sea (ICES). To ascertain the identity and the purity of the peaks in the chromatogram, 21 fractions were collected and analyzed by SS [16]; the PAHs identified prior to Shpol'skii analysis are indicated in Fig. 18.6.

Identification was achieved by comparison with reference Shpol'skii spectra [5,17]. Fig. 18.7 shows both the on-line spectra recorded at room-temperature (RT) and the Shpol'skii spectra of fraction IX. On the basis of retention time, fraction IX was identified as benzo[*b*]fluoranthene (B[*b*]F), but the on-line RT spectra (recorded at different positions of the eluting peak) indicated that at least one other compound was present. A conclusive identification, however, could not be derived from these spectra. With help of the
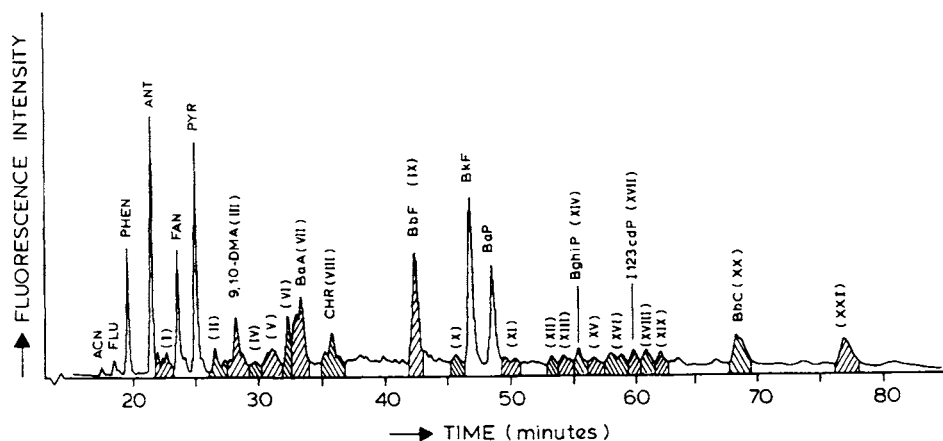
Fig. 18.6. Reversed-phase HPLC chromatogram of suspended matter sample. ACN, acenaphthene; FLU, fluorene; PHEN, phenanthrene; ANT, anthracene; FAN, fluoranthene; PYR, pyrene; 9,10-DMA, 9,10-dimethylanthracene; BaA, benz[a]anthracene; CHR, chrysene BbF, benzo[b]fluoranthene; BkF, benzo[k]fluoranthene; BaP, benzo[a]pyrene; BghiP, benzo[ghi]perylene; I123cdP, indeno [1,2,3–cd] pyrene; BbC, benzo[b]chrysene. Reproduced from [16], with permission.

Shpol'skii spectrum in Fig. 18.7d the second compound in fraction IX was identified as perylene.

Neither the chromatographic peak shape (Fig. 18.6), nor the room-temperature spectra gave any indication that fraction XV contained more than one component. The Shpol'skii spectra, however, show that not only dibenz[ah]anthracene (DB[ah]A) is present, but also some unidentified components (see Fig. 18.8). Between the emission lines of DB[ah]A (marked with an asterisk), several other peaks can be distinguished. The spectra illustrate that, because of the line-narrowing effect obtained with Shpol'skii spectroscopy, even compounds emitting in the same wavelength region will seldom show spectral overlap.

The composition of fraction XX is of special importance, since it contains benzo[b]-chrysene, B[b]C, added as an internal standard. Obviously, it is crucial to measure such a standard without interferences. The high-resolution spectra, obtained from fraction XX, are shown in Fig. 18.9, together with a reference spectrum of B[b]C. Fraction XX does not only contain benzo[b]chrysene but at least three other species are present. Unfortunately, only one could be identified, i.e. anthanthrene (ATT), for the two other species no reference spectra were found. We conclude that one should be careful applying benzo[b]chrysene as an internal standard for HPLC/fluorescence purposes, as it will probably be impossible to remove all interferences spectroscopically.

The results of the qualitative analysis of the 21 fractions [16] are summarized in Table 18.1; the number of identified compounds is roughly doubled by invoking SS. Compounds identified in the present study are underlined; an asterisk indicates that a Shpol'skii spectrum was obtained but that no matching reference spectrum was available. The latter aspect underlines the potential of the method; once a larger library of reference spectra is available, the identification possibilities of the Shpol'skii method will be greatly enhanced.

An obvious disadvantage of the Shpol'skii technique is that it can only be practiced in
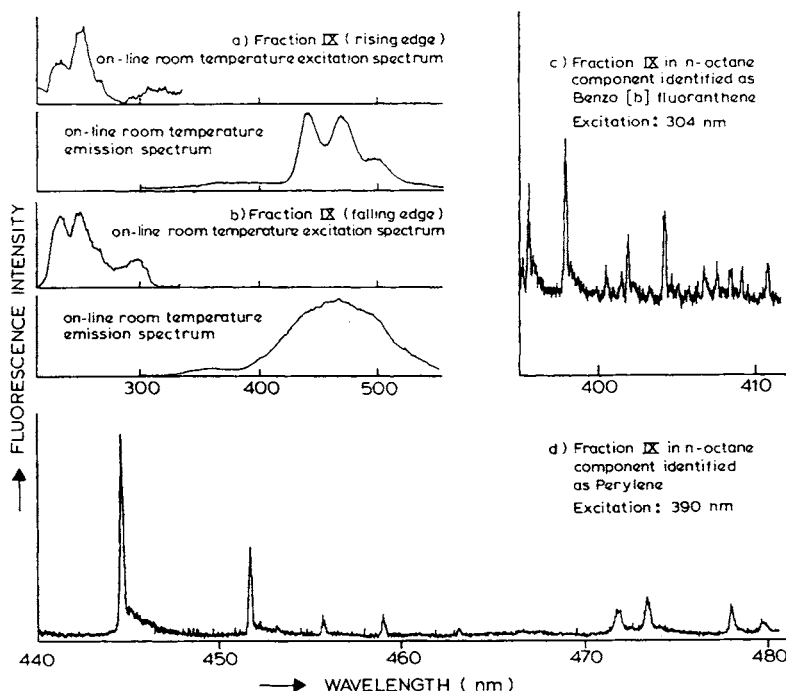
Fig. 18.7. On-line room temperature spectra (a,b) and off-line Shpol'skii spectra (c,d) of fraction IX (Shpol'skii matrix *n*-octane). Reproduced from [16], with permission.

an off-line mode. It should be emphasized, however, that the analysis of the separate eluting fractions needs only be carried out once for a particular type of sample. The results of this procedure can be used for a (qualitative) validation of the routine HPLC analysis.

18.3.1.2 Analysis of sediment reference materials

Although the advantages of the Shpol'skii method for the unambiguous identification of closely related compounds is generally recognized [16,18], it was believed that specific features of the method would preclude quantitative applications [19]. For some analyte–solvent combinations the shape and intensity of the quasilinear Shpol'skii spectra may depend rather critically on various experimental parameters, such as matrix purity, cooling rate, and sample holder design [5,9]. For most PAHs of the EPA priority pollutant list, however, reproducible Shpol'skii spectra can be obtained in *n*-octane matrices [20], provided the sample holder and cooling regime is designed for instantaneous solidification [9]. If an appropriate internal standard is used, various experimental sources of error, such as solvent evaporation, the presence of air bubbles or cracks in the frozen sample, and variations in sample thickness, optical alignment or excitation energy, are adequately corrected for.

Here, it will be shown that SS can be successfully applied to PAH analysis in sediments. Reference material HS-4, originating from a polluted harbor in Nova Scotia, Canada, was obtained from the National Research Council Canada (NRCC) [2]. The crude Soxhlet
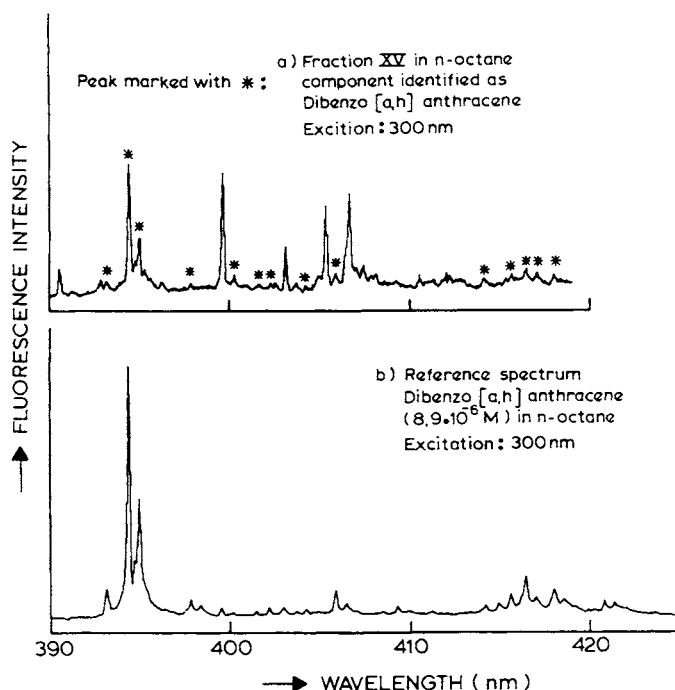
Fig. 18.8. Shpol'skii spectra of (a) fraction XV in n-octane and (b) dibenz[ah]anthracene standard
$(8.9 \times 10^{-6}$ M). The peaks marked with an asterisk are attributed to DB[ah]A. Reproduced from
[16], with permission.

extract, containing the PAHs at ca. 10^{-6}–10^{-7} M concentrations, was diluted 1:1 with a
2×10^{-7} M solution of perdeuterated pyrene (internal standard) in n-octane. Hexane was
carefully evaporated in a gentle stream of nitrogen and replaced with n-octane, a suitable
matrix for most PAHs with 4–6 fused rings [5]. Samples were cooled to 26 K and analyzed
using lamp-excited Shpol'skii spectroscopy.

Peak areas were divided by that of the internal standard and compared to previously
determined calibration plots. In order to correct for matrix transmission losses in crude
extracts (excitation light and emission from the analyte may be absorbed by the matrix, not
necessarily to the same extent as for the internal standard), an absorption spectrum of the
extract was recorded. The actual fluorescence intensity I, compared to the intensity I_n that
would be observed in a transparent n-octane matrix, can be calculated from the matrix
absorptions $A_{\lambda ex}$, $A_{\lambda em}$ at the excitation and emission wavelength of each compound:

$$\frac{I}{I_n} = \frac{1 - 10^{-(A_{\lambda ex} + A_{\lambda em})}}{(A_{\lambda ex} + A_{\lambda em})\ln 10}$$

(front-face geometry; see [21]). In practice, absorption of emission light was negligible;
correction factors for absorption of excitation light were typically not larger than 10–20%
for the PAHs analyzed in these samples.

The HS-4 extract was not only analyzed using calibration graphs, a standard addition
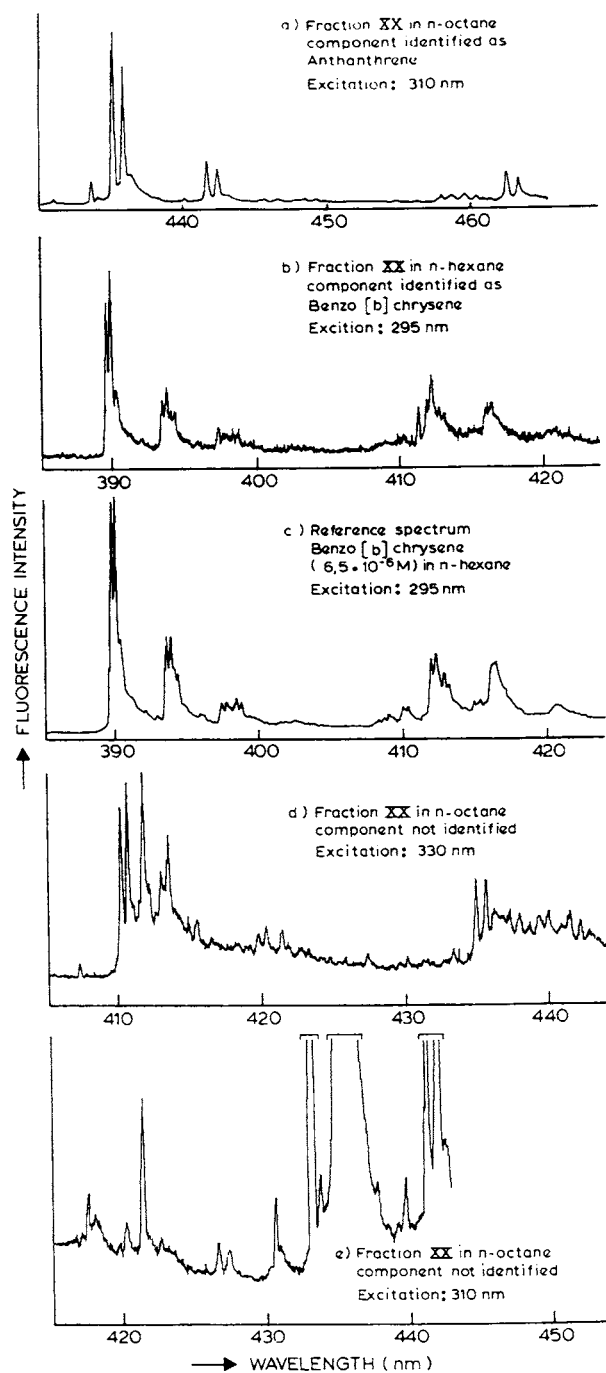
Fig. 18.9. Shpol'skii spectra of fraction XX (a,d,e in *n*-octane; b in *n*-hexane) and of benzo[*b*]chry-sene standard solution (6.5×10^{-6} M in *n*-hexane) (c). The strong emission lines in e are attributed to anthanthrene. Reproduced from [16], with permission.

TABLE 18.1

POLYCYCLIC AROMATIC HYDROCARBONS IN SUSPENDED MATTER SAMPLE IDENTIFIED WITH SHPOL'SKII SPECTROSCOPY (FROM [16])

Fraction	PAH	Fraction	PAH
I	*4,5-Methylenephenanthrene*	XIII	[a]
II	*Triphenylene*	XIV	Benzo[*ghi*]perylene
V	[a]	XV	*Dibenz[ah]anthracene*[a]
VI	*Benzo[b]fluorene*	XVI	[a]
VII	Benz[*a*]anthracene	XVII	Indeno[1,2,3–*cd*]pyrene
	2-Methylpyrene[a]		
VIII	Chrysene[a]	XVIII	[a]
IX	Benzo[*b*]fluoranthene	XIX	[a]
	Perylene		
X	*Dibenz[ac]anthracene*[a]	XX	Benzo[*b*]chrysene
			Anthanthrene[a]
XI	*Dibenz[aj]anthracene*[a]	XXI	Dibenzo[*bk*]fluoranthene[a]

[a] Compound showed quasilinear spectrum, but could not be identified. Compounds in italics were identified in this study. No Shpol'skii spectra were obtained from fractions III, IV and XII.

approach was also employed: a synthetic mixture, containing all PAHs to be determined at equal concentrations, was added to the sample at 9×10^{-8}, 3×10^{-7} and 9×10^{-7} M. The internal standard concentration was 1×10^{-7} M in all solutions. The analyte concentration in the original sample was calculated from the intercept, using simple linear regression. The results collected in Table 18.2 show excellent agreement between the two methods, indicating that the calibration curves, determined in clear standard solutions, are also applicable to crude sediment extracts. After correction for matrix absorption, the slope of the calibration plots did not differ from that of the standard addition plots for the compounds listed in Table 18.2. Since the standard addition method requires several measurements for each sample, the calibration graph method was obviously preferred.

TABLE 18.2

PAH CONCENTRATIONS (μg/g) IN SEDIMENT REFERENCE MATERIAL HS-4

PAH[a]	HPLC-Flu[b]	Shpol'skii; calib.[c]	Shpol'skii; st. add.	NRCC values[d]
Pyr	0.69 ± 0.06	0.91 ± 0.04	0.88	0.94 ± 0.12
BaA	0.39 ± 0.02	0.44	0.46	0.53 ± 0.05
Chr	0.48 ± 0.09	0.38	0.39	0.65 ± 0.08
BaP	0.43 ± 0.04	0.53 ± 0.02	0.49	0.65 ± 0.08
BbF	0.48 ± 0.04	0.68	0.66	0.70 ± 0.15
BkF	0.28 ± 0.03	0.35 ± 0.03	0.33	0.36 ± 0.05
BgP	0.37 ± 0.06	0.40	0.39	0.58 ± 0.22

[a] Pyr, pyrene; BaA, benz[*a*]anthracene; Chr, chrysene; BaP, benzo[*a*]pyrene; BbF, benzo[*b*]fluoranthene; BkF, benzo[*k*]fluoranthene; BgP, benzo[*ghi*]perylene.

[b] Results of 3 determinations.

[c] Results of 1–4 determinations.

[d] Uncertainties represent 90% confidence limits.

The data collected in Table 18.2 are within or just below the 90% confidence interval stated by the NRCC except for chrysene. There seems to be a slight negative bias, which could be the result of incomplete extraction. The HPLC–fluorescence results, obtained at the Tidal Waters Division using the same extracts, also agree reasonably well with the reference values, although the negative bias is more pronounced.

Summarizing, we conclude that lamp-excited Shpol'skii spectrometry is a suitable technique for the quantitative analysis of a range of PAHs. Measurements could be carried out on crude Soxhlet extracts without further cleanup, thus reducing sample handling time and the risk of introducing contamination.

As a rule, 'true' PAH concentrations in reference materials are determined by employing a spectrum of different analytical techniques. Since the selectivity of the Shpol'skii technique is based on spectral rather than physical separation, it is strongly recommended to include this fully independent method in quality control procedures.

Laser-excited Shpol'skii Spectrometry (LESS) with time resolved fluorescence detection has recently been developed. An important application for PAHs is the direct ultra-sensitive determination of the most potent carcinogen known to date dibenzo[a,l]pyrene (DB[a,l]P). Even the presence of this compound at 100 times lower concentration than B[a]P is already relevant. LESS offers the possibility of a pulsed site selective excitation to reduce the role of interferences and to prove the presence of different sites for one particular analyte. Moreover, application of time-resolved detection improves the signal to background ratio.

In Fig. 18.10 the Shpol'skii spectra for a standard solution of DB[a,l]P in n-octane at 26K are depicted using lamp (322 nm) and laser (411.9 nm) excitation. It is clear from the lamp spectrum that DB[a,l]P is present in two sites. With selective laser excitation the fluorescence spectrum attributed to only one separate site will be observed. In the case of DB[a,l]P, laser excitation at 411.9 nm probes the long-wavelength site, and laser excitation at 411.2 nm the site at shorter wavelength. The observation of these two sites, at these particular laser excitation wavelengths as presented in Fig. 18.10 unambiguously identifies the compound as DB[a,l]P, even if present at 0.01 mg/kg DW level in complex soil and
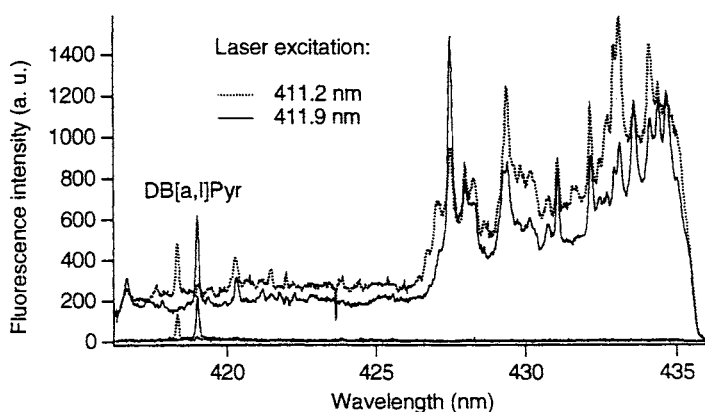
Fig. 18.10. LESS spectra of a crude extract of a floodplain soil sample (upper curves), and of a standard solution of dibenzo[a,l]pyrene (2.4 nM, lower curves). Time-resolved detection (100 ns delay), n-octane, $T = 26$ K.

sediment samples, which contain other PAHs in much higher amounts [22]. More examples of the advantages of laser excitation will be given below.

18.3.1.3 Analysis of biota

The analysis of PAHs in biota is more difficult than in sediment samples, owing to the low concentrations of the analytes and the high levels of potentially interfering substances. Nevertheless, using the Shpol'skii technique, a number of PAHs could be determined in a complex biotic extract without prior chromatographic separation [21,22]. The bioaccumulation of PAHs in living organisms is used to obtain time-integrated levels of the total biologically available fraction of these compounds in the area. The latter method is called active biological monitoring, and mussels have been demonstrated to be particularly useful for this purpose ('mussel watch'; [23]). A mussel constantly pumps and filters large amounts of sea water (ca. 50 l/day), and PAHs accumulate in the fatty tissues until the uptake is balanced by excretion and degradation and a plateau value is reached. Also detection of PAHs in other biota tissues is of interest for biologists and environmental chemists.

After removing the shell, mussels were homogenized, freeze-dried, extracted with *n*-hexane in a Soxhlet apparatus and evaporated to dryness. Terns were plucked, freeze-dried, homogenized, freeze-dried again, extracted with hexane and evaporated to dryness. Of the fatty, brownish samples thus obtained, half was simply diluted with *n*-octane (Baker analyzed grade) and measured without further cleanup. The remaining part of the extracts was further cleaned over a silica gel column (15 g, deactivated with 5% water), and eluted with hexane. For the tern sample, this washing procedure had to be repeated several times in order to obtain a sufficiently clear extract. Before measurement a known concentration of internal standard, perdeuterated pyrene, was added, and hexane was replaced with octane through selective evaporation in a stream of nitrogen. Attempts to obtain Shpol'skii spectra directly with the concentrated fatty extracts were unsuccessful; optimum results were obtained after diluting the sample 100-fold with octane. Although the background noise was not much higher than for neat academic solutions, the detection limits were of course greatly affected by the necessity of dilution. Nevertheless, it was still possible to determine benzo[*a*]pyrene, benzo[*k*]fluoranthene and pyrene in both samples [21]; the results are summarized in Table 18.3. Fig. 18.11a shows part of the tern emission spectrum, exhibiting the main fluorescence peaks of pyrene and the internal standard.

One could imagine that the Shpol'skii analysis of biota samples would benefit from a sample cleanup. Using this procedure, the fatty components were largely removed and the samples became virtually transparent in the visible and near-UV regions. Strong dilution was no longer necessary, and much larger signals could be obtained in this way. This is illustrated by the spectrum for the cleaned tern sample in Fig. 18.11b, as compared to that of the crude sample in Fig. 18.11a. The samples were checked for ten different PAHs, of which seven could be determined (see Table 18.3). Benzo[*b*]fluoranthene, benzo[*e*]pyrene and indeno[1,2,3–*cd*]pyrene could not be detected. For pyrene, the standard deviation was found to be 4.7% over seven independent measurements. For all compounds, the repeatability was better than 10%.

The results indicate that SS is appropriate for the direct determination of PAHs in fatty biotic samples. Fatty acids and other kinds of non-polar compounds hardly interfere with

TABLE 18.3

DETERMINATION OF PAHs IN CRUDE AND NEAT SAMPLES[a]

PAH	Tern before cleanup	Tern after cleanup	Mussel before cleanup	Mussel after cleanup
Benz[a]anthracene		8		40
Benzo[a])pyrene	9 (10)	4	27	17
Benzo[ghi]perylene		17		22
Benzo[k]fluoranthene	11 (11)	7	36	18
Chrysene		13		76
Pyrene	156 (153)	143	256	265
Perylene		2		8

[a] Values are in ng/g fresh weight; data in parentheses were measured using standard addition.

the measurements, as long as their total content is not much larger than 1% after dilution with octane. Of course, the dilution step leads to a severe decrease in sensitivity, but nevertheless three different aromatic compounds could be detected at the ng/ml level in the crude extract with the lamp-excited Shpol'skii method. Again, the detection potential can be expected to be considerably improved if a laser is used for excitation instead of a xenon arc lamp, in other words by applying laser-excited Shpol'skii spectroscopy (LESS).

Depending on the laser system applied there are three important advantages compared to lamp excitation [24]:

(1) High excitation power. Compared to the light intensity of a xenon arc lamp dispersed by a high-throughput monochromator, an Nd-YAG/dye laser combination may offer an increase in excitation power of typically two orders of magnitude. Furthermore, the laser
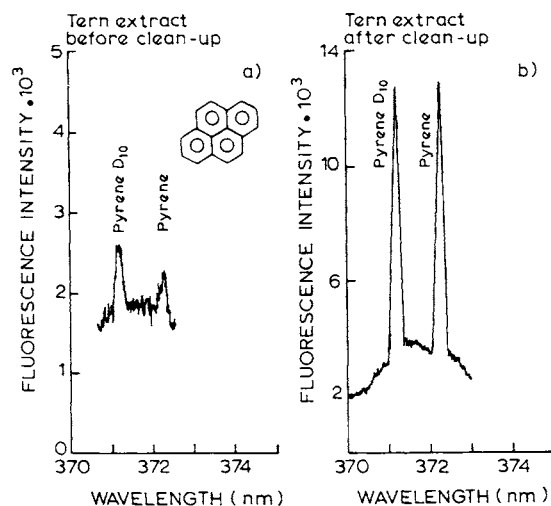
Fig. 18.11. Part of the Shpol'skii fluorescence spectra of the tern sample; lamp excitation, 335 nm; temperature, 26 K. (a) Before cleanup; [pyrene–d_{10}] = 3×10^{-8}M; (b) after cleanup; [pyrene–d_{10}] = 1×10^{-7}M. Reproduced from [21], with permission.
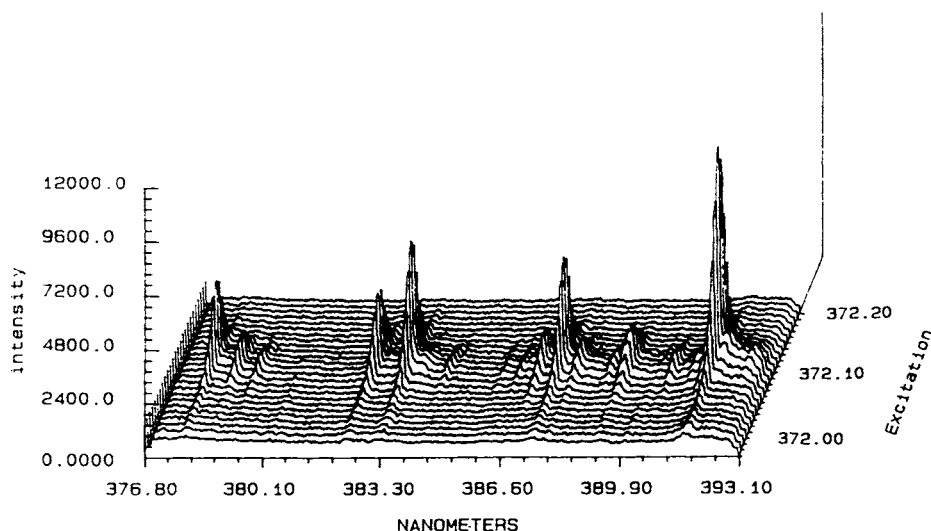
Fig. 18.12. Excitation-emission plot of pyrene in *n*-octane at 28 K; concentration 5.0×10^{-7} M, laser excitation. Reproduced from [24], with permission.

beam can easily be focused on a very small (microliter or less) sample volume. This is important, as in high-resolution spectroscopy the fluorescent spot has to be projected onto the very narrow entrance slit of the emission monochromator. Overall, we can obtain a total increase in the effective excitation power of three orders of magnitude.

(2) High monochromicity. Since the S_1–S_0 region of the Shpol'skii absorption spectrum is also narrow-banded [5], it is possible to selectively excite one particular compound in a mixture. Fig. 18.12 shows a three-dimensional excitation–emission spectrum of pyrene in *n*-octane [2]. Emission spectra were continuously recorded as the laser was tuned through the 0–0 transition. Pyrene-d_{10} was also present in the mixture, but although its excitation wavelength is shifted over only one nanometer, it is completely invisible, as the laser line does not match exactly. It is clear that we have here a powerful tool to increase the emission of a particular analyte and at the same time reduce interferences from other compounds. Fig. 18.13a shows the pyrene emission from the crude tern extract using laser excitation.

(3) Time-resolved detection. if the laser system is of the pulsed type, we can discriminate between the relatively long-living (20–500 ns) emission of the aromatic analytes and instantaneous processes like stray-light or (Raman) scattering. Also short-living background luminescence is removed. A pulse generator was used to activate the intensifier photocathode of the diode-array detector 50 ns after the laser shot. The effect of time-resolution is illustrated if we compare the signal-to-noise ratios in Fig. 18.13a,b. Of course, for shorter-living analytes a shorter delay must be used and the background noise may not be removed completely.

The overall spectral improvement due to laser excitation is clearly demonstrated if we compare Fig. 18.13b to Fig. 18.11a, showing the pyrene emission from the same crude tern extract. The advantages are obvious: both sensitivity as well as selectivity are remarkably improved.
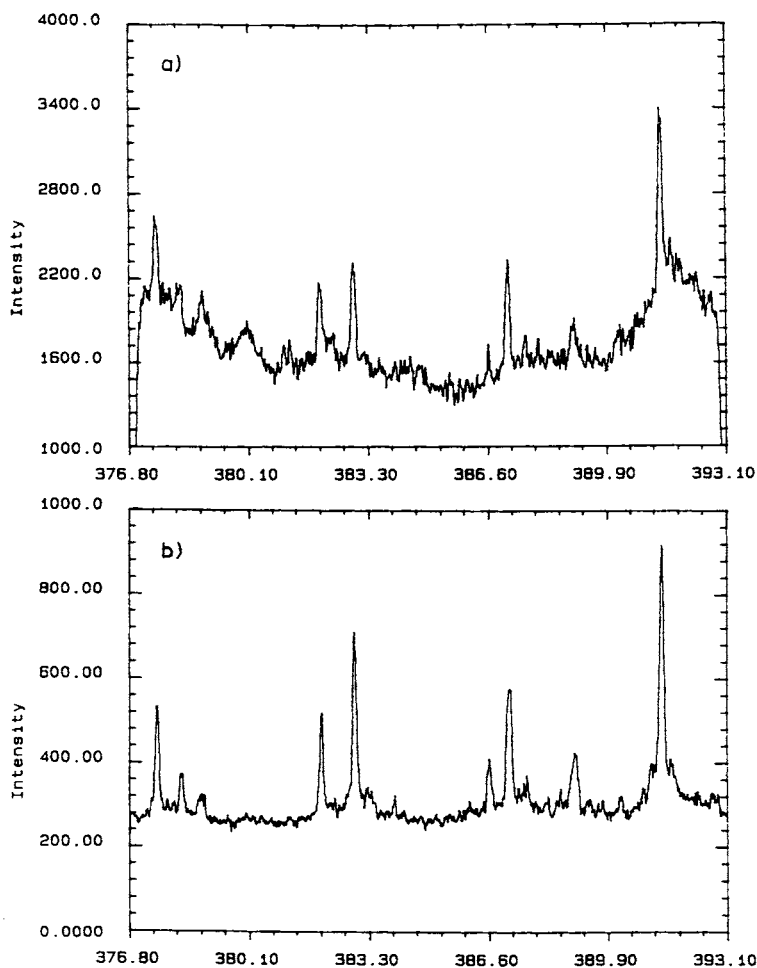
Fig. 18.13. Shpol'skii emission of pyrene in crude tern extract in *n*-octane at 28 K; laser excitation 372.10 nm. (a) No time resolution; (b) with time-resolved detection (delay = 50 ns). Reproduced from [24], with permission.

To summarize, SS can be used to qualitatively and quantitatively determine PAHs in environmental samples [2,20–22]. The technique is very sensitive: the limit of detection for pyrene (in a synthetic mixture of 10 PAHs in *n*-octane using conventional lamp excitation) is as low as 5×10^{-10} M and for benzo[*a*]pyrene with laser excitation a detection limit of 5×10^{-12} M has been obtained (for a 10 µl sample volume this means 50 attomoles [2]). For trace level analysis of PAHs in complex samples, it will be essential to employ laser excitation. Of course, one should realize that laser systems offering acceptable powers in the near-UV region are still quite expensive, and an extensive wavelength range can not be reached without having to change the dye solution or the system configuration. Nevertheless, for a number of complicated analytical problems (laser-excited) Shpol'skii spectroscopy will offer an adequate solution.

18.3.2 Shpol'skii spectrofluorimetric analysis of PAH metabolites

In order to monitor exposure to PAHs in the environment, the determination of their concentrations in the various environmental compartments may not be sufficient, since bioavailability is not taken into account. Biological monitoring, that is, the determination of a particular compound (or its metabolites) in a specific organism or tissue, can provide valuable information on the actual uptake rate [25]. Fish usually do not show considerable accumulation of PAHs [26]. Upon absorption, PAHs are rapidly metabolized into more polar derivatives that are stored in the gallbladder to be excreted [27,28]. Attempts to biomonitor PAH uptake by fish should therefore concentrate on PAH metabolites in excreta rather than on parent PAHs in tissue. Krahn and coworkers [29] developed HPLC/fluorescence and GC-MS methods to determine PAH metabolites in fish bile.

The potent carcinogen benzo[*a*]pyrene (BaP) is often used as a model compound to study the toxic effects of PAHs [30]. BaP is metabolized by hepatic enzyme systems into a number of mono- and polyhydroxylated derivatives. Some reactive species may form adducts with proteins or DNA, but most metabolites are rapidly excreted in the form of glucuronide, sulfate, or glutathione conjugates [31]. In laboratory experiments, the biotransformation products of BaP are usually analyzed by means of HPLC; to avoid problems with detection sensitivity, toxicologists can administer high doses of BaP or use radioactive material. In the field, the concentration of BaP metabolites in bile of feral fish could be used as an indicator of exposure to BaP and related PAHs in the area, but the detection of BaP metabolites requires extremely sensitive and selective methods. Bile of fish exposed to many different PAHs will contain an even more complex mixture of PAH metabolites that may interfere with the analysis. Using HPLC/fluorescence, Krahn and colleagues [32] succeeded in detecting 3-hydroxy-BaP (3-OH-BaP) in some bile samples from a highly polluted site near Seattle, but sub-ppb sensitivity would be needed for the detection of BaP exposure in other, less polluted areas. As an extra complication, the bile volumes available are usually not sufficient for trace enrichment.

Shpol'skii spectrometry may offer the required sensitivity and selectivity, but PAH metabolites will be less compatible with the matrix than their parent compounds because of their increased polarity. Weeks and coworkers [33] described a procedure to transform monohydroxy-benz[*a*]anthracenes into less polar methoxy derivatives, which could subsequently be analyzed by means of LESS. Recently, the same research group reported the derivatization and Shpol'skii spectra of a wide range of BaP metabolites: monohydroxy-BaP derivatives, BaP-dihydrodiols, BaP-dihydrodiolepoxide, as well as BaP-tetrahydrotetrol [34].

The practical applicability of the Shpol'skii technique to the analysis of BaP metabolites in fish bile has been shown in the literature [2,35]. An analytical protocol was developed for the quantitation of 3-OH-BaP in bile samples. The model fish studied was the flatfish species flounder (*Platichthys flesus*). Exposure to BaP was realized following two different methods. High exposure levels were realized by administering a single dose of BaP (parenteral injection in acetone/Mulgofen 620; 0.78 or 4.04 mg/kg body weight). The fish were fed shrimp (*Crangon crangon*) until 2 days before injection and were sacrificed 48 h after injection. To simulate semi-chronic exposure to realistic BaP pollution levels, flounders were kept during four weeks in three different mesocosms: (1) moderately polluted Rotterdam harbor sediment (dredging class II, direct contact with the sediment

was possible); (2) indirect exposure to Rotterdam harbor sediment (Wadden Sea sand bottom; food and water equilibrated with the polluted harbor sediment; (3) Wadden Sea sand bottom (control group). The PAH contents of the sediments (fine fraction only) were determined after wet sieving over a 63 μm nylon filter, by means of HPLC with fluorescence detection [36]. The harbor sediment contained 450 ppb BaP and 800 ppb pyrene; the Wadden Sea sand contained 140 ppb BaP and 180 ppb pyrene (ng/g dry weight of fine fraction). The fish were fed until 2 days before section to allow the accumulation of metabolites in the gallbladder and to reduce the confounding effects from different feeding habits.

Bile was collected from the gall bladder by means of a syringe and stored in vials in the dark at $-20°C$ until further use. The bile samples were treated as follows: 20 μl of bile was diluted with water to 1 ml and incubated for 2 h at 37°C with 20 μl of β-glucuronidase/aryl sulfatase solution to hydrolyze conjugated metabolites. Typically, maximum yield was reached within 20–30 min. The free metabolites were quantitatively extracted by repeated extractions with *n*-hexane (4 $\times$ 3 ml). For direct analysis of underivatized metabolites, hexane was evaporated in a stream of nitrogen and the residue dissolved in 2 ml of *n*-octane. In most cases, however, the volume of the extract was reduced to ca. 0.5 ml and the metabolites derivatized according to a procedure adopted from Weeks and coworkers [33]: 2 mg of sodium hydride was washed three times with *n*-pentane in a flask under nitrogen atmosphere, 1 ml of dimethyl sulfoxide (DMSO) was added and the mixture was stirred at 70°C for several minutes until the formation of H_2 bubbles had ceased. After cooling to room temperature, 100 μl of methyl iodide and the bile extract were added; after several minutes of stirring the reaction was quenched with 4 ml of water. The methylated products were quantitatively extracted with 2 times 3 ml of *n*-hexane. This extraction solvent was preferred over *n*-octane because of its lower boiling point and higher purity. For Shpol'skii analysis, the extract was concentrated and the solvent gradually replaced with *n*-octane in a gentle stream of nitrogen. For quantitation, perdeuterated perylene was added to the final analytical sample as an internal standard; 2×10^{-8} M for bile samples from the most polluted mesocosm, 2×10^{-9} M for the other samples.

Direct Shpol'skii analysis of monohydroxy-BaP metabolites is possible; 1-OH-BaP and 3-OH-BaP were identified in fish bile after injection with BaP [37]. However, the sensitivity of the method proved insufficient for application to the mesocosm study. These analytes are not fully compatible with the crystalline *n*-octane host. As a consequence, the actual concentration of analyte molecules trapped in crystalline sites and producing quasilinear emission is rather low and thus the achievable sensitivity is disappointing. Furthermore, the shape and intensity of the Shpol'skii spectra depend critically on concentration, cooling rate or the presence of polar impurities in the matrix, so proper quantitation in real samples is very difficult [38].

For phenolic metabolites the above mentioned derivatization reaction with methyl iodide in DMSO is appropriate to solve these problems [35]: the reaction is very rapid, practical, straightforward, and quantitative, and the methylated analytes provide good Shpol'skii spectra in *n*-octane with the possibility of isomer-specific determination (see Fig. 18.14). To illustrate the increase in sensitivity: in case of 3-OH-BaP, the improved host-guest compatibility after derivatization resulted in a 20-fold increase in quasilinear fluorescence intensity; the detection limit improved to 5.0×10^{-10} M (lamp excitation at 300 nm).
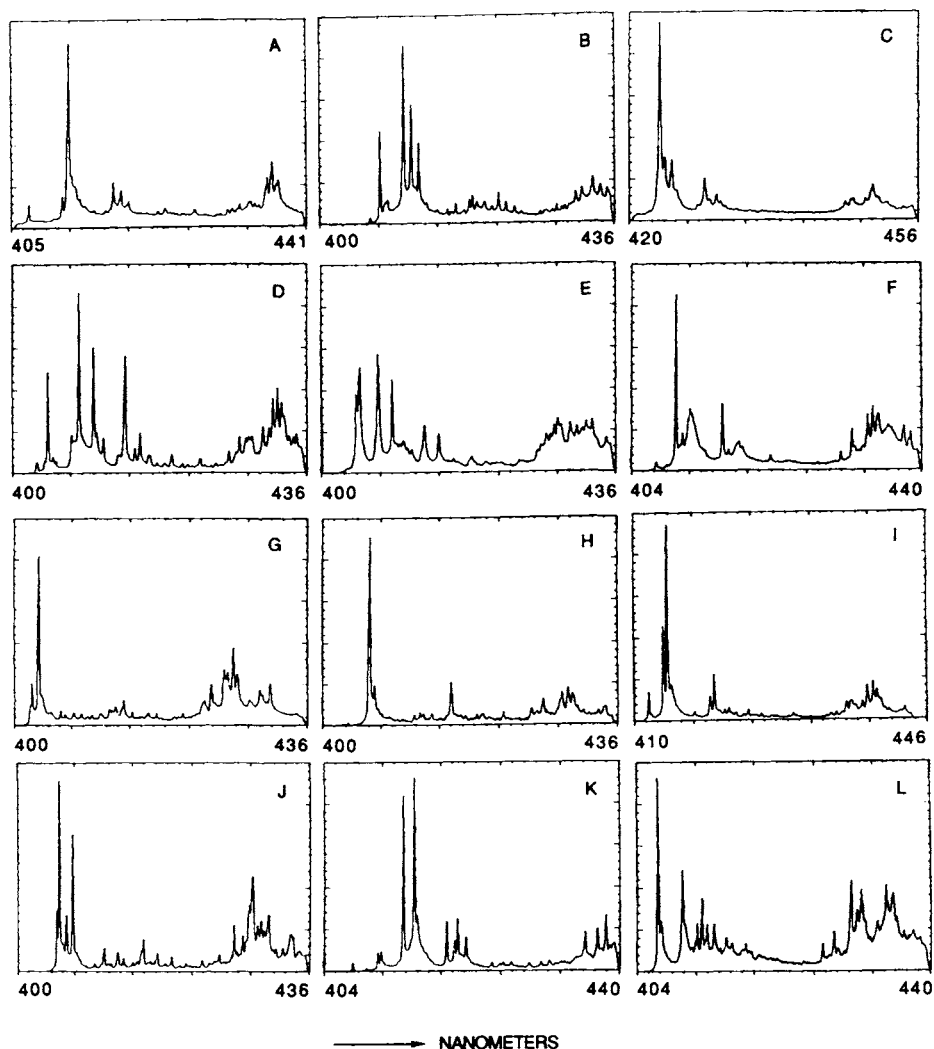
Fig. 18.14. Shpol'skii spectra in n-octane of phenolic BaP metabolites after derivatization, using nonselective laser excitation at 348 nm. (A) 1-methoxy BaP, 10^{-7} M; (B) 2-methoxy BaP, 10^{-6} M; (C) 3-methoxy BaP, 2×10^{-7} M; (D) 4-methoxy BaP, 10^{-6} M; (E) 5-methoxy BaP, 10^{-6} M; (F) 6-methoxy BaP, 10^{-6} M; (G) 7-methoxy BaP, 10^{-6} M; (H) 8-methoxy BaP, 10^{-6} M; (I) 9-methoxy BaP, 10^{-6} M; (J) 10-methoxy BaP, 10^{-6} M; (K) 11-methoxy BaP, 10^{-6} M; (L) 12-methoxy BaP, 10^{-6} M. Reproduced from [35], with permission.

A typical Shpol'skii spectrum of a methylated bile extract (originating from fish that had received a high dose via injection) is shown in Fig. 18.15. Nonselective laser excitation at 348 nm was employed, in order to be able to determine all metabolites simultaneously; the overall dilution factor was 1000. The spectrum is dominated by 3-methoxy BaP and 1-methoxy BaP (compare with reference spectra in Fig. 18.14a,c); the relative contribution of the latter varied considerably between individuals: between 7% and 26% of the
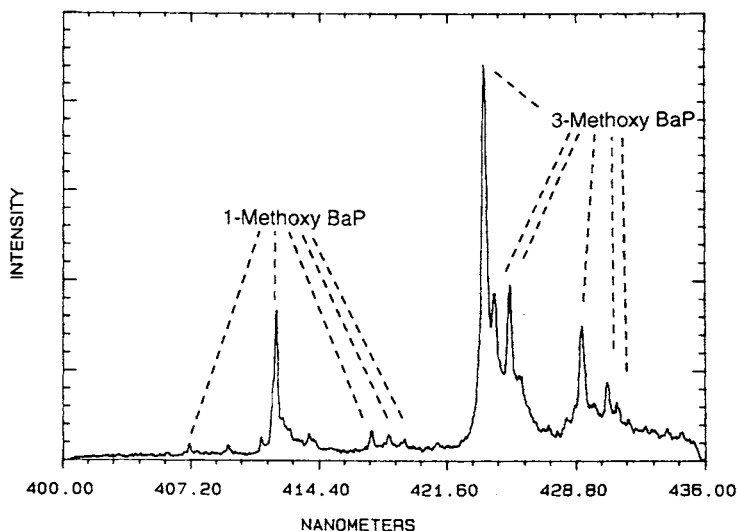
Fig. 18.15. Shpol'skii spectrum of methylated flounder bile sample (0.78 mg/kg BaP injected), featuring 1-methoxy BaP and 3-methoxy BaP. Nonselective laser excitation at 348 nm. Reproduced from [35], with permission.

total amount of metabolites detected. The metabolite 3-OH-BaP was chosen as a marker compound for the biomonitoring of BaP uptake from a polluted environment.

For quantitation of 3-methoxy BaP with LESS, an internal standard should be excitable at the wavelength chosen for the analyte, and should have a sufficiently strong emission line in the emission window covered by the multichannel detector. Perdeuterated perylene was found to meet the above requirements; ratioing the peak areas of the 0–0 emission lines, a straight calibration curve was obtained for 3-methoxy BaP in the concentration range of interest (3×10^{-11} M to 1×10^{-8} M). The absolute detection limit (S/N = 3) for 3-methoxy-BaP, using laser excitation at 418.36 nm, was found to be 5×10^{-12} M (50 attomole) in n-octane solutions. The detection in bile extracts was not seriously affected by matrix interferences. When the sample treatment was carried out without overall dilution (provided that sufficient bile was collected), the detection limit was still 2×10^{-11} M or 0.005 ng/ml. For most samples, we used an overall dilution factor of 20; in that case the detection limit was 2×10^{-10} M or 0.05 ng/ml in the original sample, which was sufficient to detect exposure to BaP in all samples from the mesocosm experiment. The repeatability of the method (four replicates of sample extraction and determination) was 16%.

Laser-excited Shpol'skii spectrometry of 3-OH-BaP was applied to a mesocosm study in which flounders were exposed during four weeks to three degrees of pollution. Mesocosm 1 and 3 reflect the range of PAH pollution levels encountered in the Dutch coastal waters and estuaries. Mesocosm 2 was designed to find out what route of exposure contributes most significantly to the total BaP uptake. Hydrolyzed bile samples were derivatized, the methylated extracts were cooled to 23 K and their Shpol'skii spectra were recorded using selective laser excitation at 418.36 nm. For the mesocosm samples, nonselective excitation at 348 nm could not be used because of two reasons: limited sensitivity and spectral overlap with emission bands from 1-methoxypyrene (1-OH-pyrene
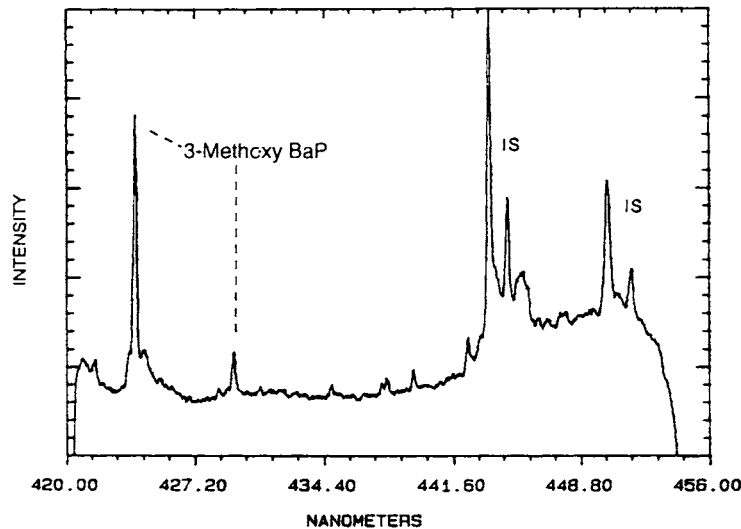
Fig. 18.16. Shpol'skii spectrum of methylated bile extract from Wadden Sea mesocosm, featuring 3-methoxy BaP. Selective laser excitation at 418.36 nm; IS, perylene d_{12}. Reproduced from [35], with permission.

is usually present at much higher levels in fish bile). Notwithstanding the 20-fold dilution caused by the sample workup, 3-methoxy-BaP could be detected in all samples, even from mesocosm 3 (Fig. 18.16). The multiplet structure from Fig. 18.14c has disappeared as the result of site-selective excitation. The analytical results, summarized in Table 18.4, show that fish exposed to Rotterdam harbor sediment had absorbed and metabolized 40 times more BaP than fish from the Wadden Sea sand basin. Furthermore, fish from the second mesocosm showed only a 6-fold increase, indicating that some uptake of BaP can take place through the water phase or through the diet [39], but that direct contact with the sediment is the major route of exposure for a bottom-dwelling fish like flounder. Direct absorption through skin or gills, or ingestion of PAH-containing particles, may both be important factors.

Table 18.4 also lists 1-hydroxypyrene levels determined by means of synchronous fluorescence spectrometry (SFS) [40]. Although 1-methoxypyrene can be measured

TABLE 18.4

BaP AND PYRENE METABOLITES IN FISH BILE AFTER EXPOSURE TO DIFFERENT MESOCOSMS [2,35][a]

	3-OH-BaP (LESS)	1-OH-pyrene (SFS)
Harbor sediment (direct contact possible)	50 ± 36 (n = 9)	15900 ± 6700 (n = 23)
Harbor sediment indirect (PAH uptake through food and/or water)	7.7 ± 2.4 (n = 4)	2600 ± 1500 (n = 16)
Sand bottom	1.2 ± 0.1 (n = 3)	800 ± 480 (n = 26)

[a] Concentrations, in ng/ml, are expressed as the arithmetic mean ± standard deviation (number of samples).

with Shpol'skii spectrometry in an *n*-octane matrix [40], the relatively high concentrations allowed the determination (in a large number of samples) with a faster, more conventional method as will be shown in the next section. The 1-OH-pyrene data show a similar trend as the results for 3-OH-BaP, but the 1-OH-pyrene concentrations are a factor of 300–600 higher, which is not explained by the relative contents of the parent PAHs in the sediments, nor by the fact that 3-OH-BaP is not the only metabolite of BaP. Apparently, the bioavailability of pyrene is much higher than that of BaP, which agrees with the kinetic studies of Landrum [25]. The standard deviations reported in Table 18.4 are an indication of the usual biological spread [27].

It was concluded that the described procedure is appropriate for the quantitative determination of 3-OH-BaP in fish bile. Employing enzymatic hydrolysis, chemical derivatization, and laser-excited Shpol'skii spectrometry, the detection limit is as low as 0.005 ng/ml, which is amply sufficient for the biomonitoring of BaP uptake in the Dutch coastal waters and in inshore areas. Some extra effort will be required to monitor the much lower levels of BaP pollution at open sea.

18.3.3 Synchronous fluorescence spectrometry of PAH metabolites

In the preceding section it was shown that direct Shpol'skii analysis of monohydroxy-BaP metabolites in fish bile is possible if chemical derivatization (methylation) is applied and laser- instead of lamp excitation is invoked. Evidently, the synchronous fluorescence spectroscopy (SFS) technique is not sensitive enough for the determination of 3-OH-BaP. However, the concentrations of 3-OH-BaP in bile, measured with Shpol'skii spectroscopy, seem to correlate with the 1-hydroxypyrene levels (see Table 18.4). Since 1-OH-pyrene is a major metabolite in bile of fish exposed to PAH polluted sediments [32], it is interesting to examine the applicability of SFS as a rapid screening technique for the determination of this compound [40].

The HPLC–fluorescence chromatogram of hydrolyzed bile from flounder (mesocosm 1, see previous section) is depicted in Fig. 18.17. The compound eluting at 4 min (RP-18 column, acetonitrile/water 70:30 v/v) was 1-OH-pyrene, unambiguously identified by Shpol'skii spectroscopy [41].

Fig. 18.18a presents a conventional fluorescence emission spectrum of diluted bile of flounder after exposure to Rotterdam harbor sediment, compared to the emission spectrum of a pyrene-1-glucuronide standard solution. The spectral features of conjugated 1-OH-pyrene are easily discerned in the total bile spectrum, but correct quantitation is hampered by interferences. Fig. 18.18b illustrates the advantage of synchronous scanning: the standard spectrum of pyrene-1-glucuronide is almost completely reduced to one single emission band. In the SFS spectrum of total bile, the emission peak attributed to pyrene-1-glucuronide is now more reliably quantitated.

The SFS spectra were recorded with a wavelength interval $\Delta\lambda$ of 37 nm. SFS analysis of parent PAHs in environmental samples is often carried out using a much smaller $\Delta\lambda$ (typically 3–5 nm, corresponding to the compounds Stokes' shift), in order to achieve maximal spectral simplification. The Stokes' shift of conjugated 1-OH-pyrene, however, is too small (only 1.5 nm) and the molar extinction coefficient of the 0–0 absorption band is too low for practical application. Using $\Delta\lambda = 37$ nm, light scattering was strongly reduced and sensitivity was optimal. The repeatability of the method was 9% (six inde-
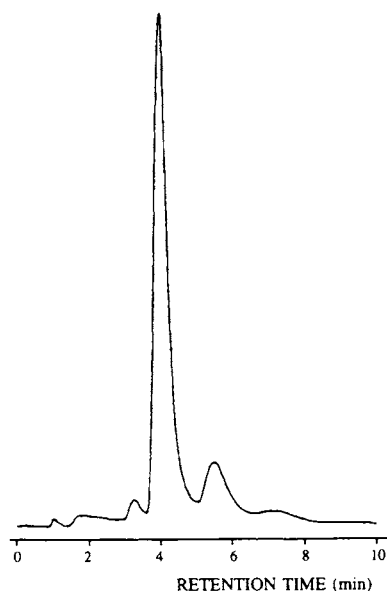
Fig. 18.17. HPLC separation of flounder bile sample from mesocosm 1, after hydrolysis and extraction with n-hexane. 1-Hydroxypyrene elutes at $t = 4$ min. Fluorescence detection 345/395 nm. Reproduced from [40], with permission.

pendent determinations of a bile sample from the reference mesocosm). The limit of detection (S/N = 3) was 0.1 ppb in the final analytical solution, which corresponds to 50–200 ppb in the original bile sample. The detection limit is directly proportional to the
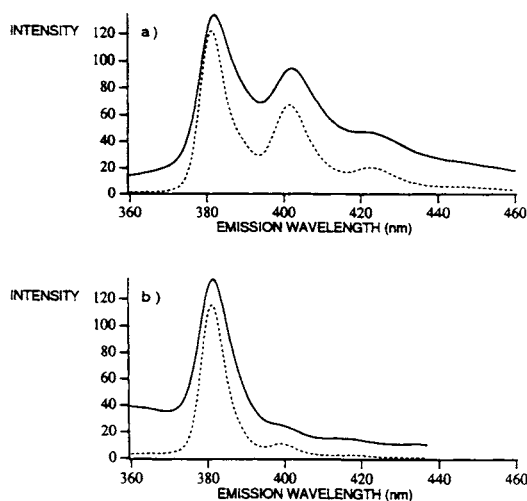
Fig. 18.18. Conventional (a, $\lambda_{exc} = 345$ nm) and synchronous (b, $\Delta\lambda = 37$ nm) fluorescence spectra in ethanol/water 50:50. Full lines: bile sample from mesocosm 1, diluted 1:2000. Dashed lines: pyrene-1-glucuronide reference standard; 5×10^{-8} M. Reproduced from [40], with permission.
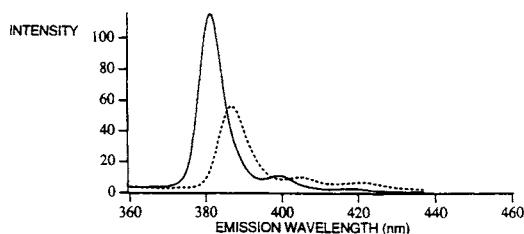
Fig. 18.19. SFS spectra in ethanol/water (50:50); $\Delta\lambda = 37$ nm. Full line: pyrene-1-glucuronide conjugate before hydrolysis. Dashed line: 1-hydroxy pyrene (hydrolysis product). Reproduced from [40], with permission.

dilution factor, required to obtain a sufficiently transparent matrix. For less deeply colored samples (i.e. in field samples from fish that are not starved prior to section), much lower detection limits can be obtained (10–20 ppb).

18.3.3.1 Calibration

Obviously, the most straightforward way to perform quantitative fluorimetric measurements would be to use a series of pyrene-1-glucuronide standard solutions for calibration. Unfortunately, this compound is, as far as we know, not commercially available. Another problem is the fact that the conjugate is quite stable at $-20°C$, but at ambient temperatures hydrolysis was observed. For these reasons, the possibility of using free 1-OH-pyrene as an alternative standard was explored. The fluorescence excitation- and emission spectra of free- and conjugated 1-OH-pyrene are rather similar in shape, but two effects must be taken into account. The spectra of the conjugate are blue shifted by 5 nm and more intense by a factor of 2.2 ± 0.1 ($n = 5$) (see Fig. 18.19). The latter phenomenon can be fully ascribed to a difference in fluorescence quantum yield. Using excimer laser excitation a fluorescence lifetime of 15 ± 2 ns was measured for free 1-hydroxypyrene, compared to 31 ± 2 ns for pyrene-1-glucuronide [40].

18.3.3.2 Validation of the method

1-OH-pyrene was determined in a number of flounder bile samples using two independent methods: SFS and HPLC–fluorescence. The concentrations ranged from 280 ng/ml (lowest value from mesocosm 3) to 27 300 ng/ml (highest value from mesocosm 1). Quantitation of the SFS intensities was carried out using standard solutions of free 1-OH-pyrene and a correction factor of 2.2. Calibration solutions in 500-fold diluted bile from a reference site yielded equal intensities as calibration solutions in clean ethanol/water, indicating that matrix absorption and other possible quenching effects had been sufficiently reduced by dilution. Chromatographic and spectroscopic techniques yielded comparable results; the concentrations determined with HPLC were on the average $93 \pm 17\%$ ($n = 14$) of the values determined with SFS. It is concluded that the determination of 1-hydroxypyrene with the rapid SFS technique can be carried out with sufficient accuracy. The precision of the method is fully adequate regarding the biological variability encountered in this type of samples.

Recent testing of the method at the North Sea and at coastal and freshwater sites in The Netherlands has revealed that the SFS method is usually sensitive enough for field monitoring purposes. 1-OH-pyrene levels close to the detection limit of SFS were measured at some remote North Sea locations only [2]. Thus, provided the PAH metabolite profile is roughly constant, monitoring of 1-OH-pyrene by SFS can be used as an indication of the local exposure levels to (combustion-related) PAHs. Whenever there are reasons to suspect a gross deviation from the usual metabolite profile (for instance in the case of exposure to creosote or petroleum), then more specific techniques like HPLC–fluorescence, GC–MS or laser-excited Shpol'skii spectrometry could be used to determine the relative contribution of other PAH metabolites. Recently, the advantages and disadvantages of these methods for fish bile analysis have been critically compared [42].

As shown above, the Shpol'skii technique has been successfully applied to parent PAHs and many nonpolar, rigid PAH derivatives. Moderately polar compounds, such as nonderivatized phenolic PAHs or amino-PAHs (see the following section) may also show quasiline spectra, but often the sensitivity is far from optimal. The Shpol'skii effect has not been observed for very polar compounds or for very flexible molecules. In such cases, an alternative method, fluorescence line narrowing spectroscopy (FLN), can be invoked. In this case the analysis is carried out directly in the disordered matrix, and the line-narrowing effect is obtained by selecting a subpopulation of isoenergetic analyte molecules by means of a narrow-banded laser tuned to a specific S_1–S_0 absorption. At low temperature this selection or isochromate is retained during the lifetime of the excited state and only this subpopulation will fluoresce, yielding a line-narrowed spectrum. This method has been applied to a variety of complex PAH derivatives, such as conjugated PAH metabolites [43], PAH–protein adducts [44] and in particular PAH–DNA adducts [45,46]. A detailed discussion of FLN spectroscopy is beyond the scope of this chapter, but some excellent reviews have been published [47,48]. The FLN technique is most often used for identification, but it can also provide information on three-dimensional structures [49,50].

18.3.4 Shpol'skii spectrofluorimetric analysis of nitrogen-substituted PAHs

In the first part of this section attention will be confined to amino- and nitro-PAHs, compounds that have attracted wide interest in the analytical chemistry literature and that have been quite recently studied by Shpol'skii spectroscopy as well [5]. In the second part some recent results on nitrogen-containing in-ring substituted PAHs will be evaluated, compounds usually denoted as azaarenes and carbazoles [52].

18.3.4.1 Amino- and nitro-PAHs

The analytical interest for amino- and nitro-PAHs is undoubtedly due to the fact that these compounds induce strong mutagenic effects when administered to living organisms, while they have shown to be present in a wide variety of environmental matrices such as coal-derived products, shale oil and synthetic fuels and diesel and gasoline exhaust aerosol particles. Furthermore, they may play a role in the environmental fate of PAHs. By now, various robust analytical techniques are available to detect these types of compounds,

mainly based on capillary gas chromatography (GC) coupled to mass-spectrometric (MS) or nitrogen-selective detectors, as well as on-column liquid chromatography (LC) followed by electrochemical, absorption, fluorescence or chemiluminescence detection. For this reason, Shpol'skii spectrofluorimetry will only have the potential to become an alternative approach, provided that it enables the identification of individual amino- and nitrocompounds (including discrimination between isomeric ones) at sufficiently low concentration levels in complex environmental samples.

The problem faced here is that amino- and nitro-PAHs are far more difficult to handle in Shpol'skii fluorimetry than their parent compounds. This explains why significant progress in this field has only been reported quite recently, as will be obvious from a recent review paper [53]. Nitro-PAHs are essentially nonfluorescent. They are only amenable to Shpol'skii fluorimetry after applying chemical reduction which converts them to the corresponding amino-PAHs. Compounds of the latter class exhibit strong fluorescence, maybe even stronger than the parent PAHs. However, as far as high-resolution luminescence is concerned, the presence of the NH_2 substituent causes some problems. First of all, aromatic amines tend to form aggregates, as also observed for hydroxy-substituted PAHs; a phenomenon that deteriorates the Shpol'skii spectrum. It should be prevented as much as possible by making the time needed to solidify the sample as short as possible; for modern equipment which provides efficient heat-exchange, it is shorter than 1 s. Secondly, aromatic amines are prone to electron-transfer mechanisms and therefore light-sensitive. Consequently, the duration of light exposure and spectral data acquisition is limited to 1–20 s, depending on the signal intensity and, furthermore, the use of an intensified diode array enabling simultaneous fluorescence emission detection is of crucial importance. Thirdly, in view of the mesomeric effect of the amino group, for aromatic amines the electron–phonon coupling is much stronger than for parent PAHs, while the Shpol'skii effect is based on a weak coupling. This explains why for some compounds, as for instance 1-aminoanthracene and 3-aminofluoranthene, poor spectra are obtained, dominated by the presence of phonon wings. Furthermore, it is clear that for amino-PAHs the choice of the particular solvent to form a matrix is rather critical and that even minor amounts of impurities play a significant role.

As a consequence of the three factors discussed above it should be stated that the Shpol'skii technique is not generally applicable to the detection of the whole range of amino-PAHs and that the detection limits are less favorable than for parent PAHs. Nevertheless, interesting progress has been achieved. This is exemplified by Fig. 18.20 which shows the Shpol'skii spectra of reduction products of nitro-PAHs obtained after chemical reduction of the standard reference material SRM 1587 (comprising seven nitro-PAHs). The presence of 6-nitrochrysene, 1-nitropyrene, 6-nitrobenzo[*a*]pyrene, 7-nitrobenz[*a*]anthracene and 2-aminofluorene (not shown) could be readily confirmed, whereas spectra from reduced 9-nitroanthracene and 3-nitrofluoranthene could not be recorded. Also the analysis of a standard diesel particulate material, denoted as SRM 1650, was successfully carried out. The standard material SRM 1587, mentioned above, was used for spiking purposes. As illustrated by Fig. 18.21, the method enables the determination of 1-nitropyrene in the diesel material present at a level of about 19 μg/g.

To summarize, Shpol'skii spectroscopy has some potential as a complementary analytical tool for the determination of a number of individual amino- and/or nitro-PAHs. Its applicability might be increased if sample solidification could be significantly accelerated.

Another possibility is to develop an appropriate chemical derivatization of the amino-PAHs as has also been applied to hydroxy-PAHs [33–35].
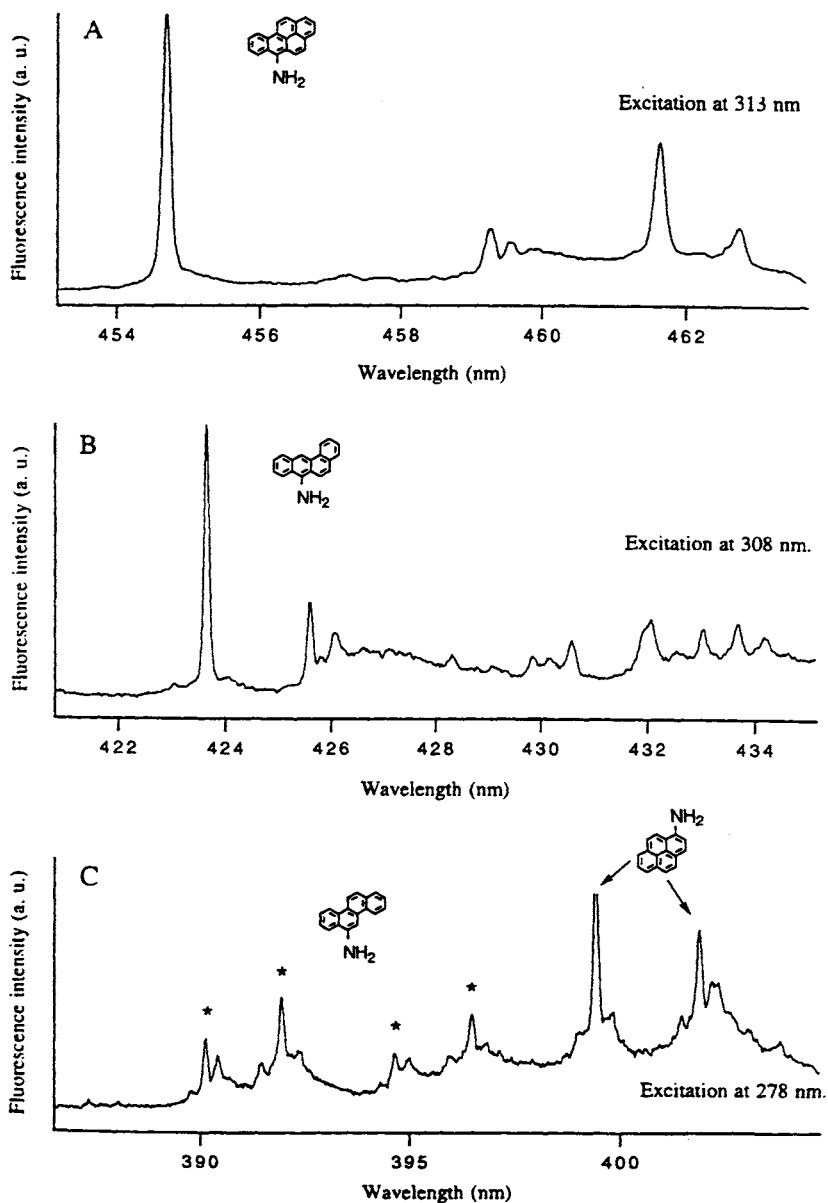
Fig. 18.20. Shpol'skii fluorescence spectra of reduction products of nitro-PAHs obtained after chemical reduction of SRM 1587 (1:10 dilution) with zinc powder: 6-aminobenzo[a]pyrene (A), 7-aminobenz[a]anthracene (B), 6-aminochrysene (indicated with asterisks) and 1-aminopyrene (C). $T = 25$ K, n-octane. Reproduced from [51], with permission.
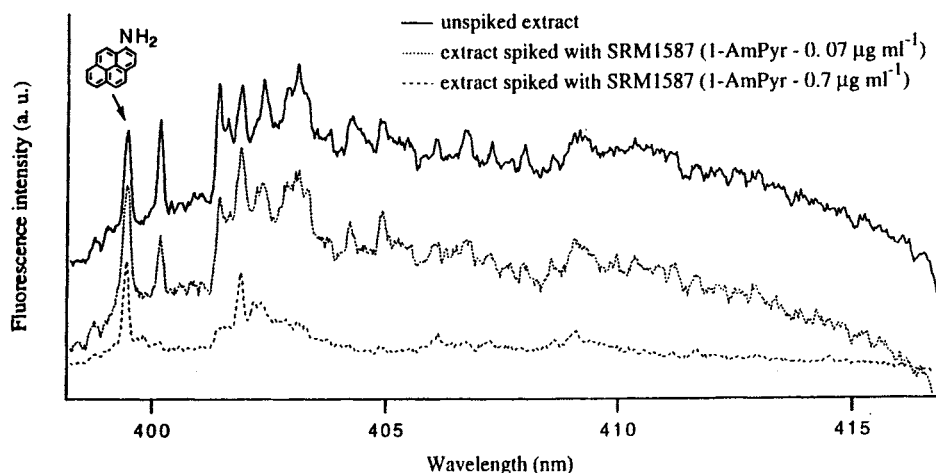
Fig. 18.21. Identification of 1-aminopyrene by means of Shpol'skii fluorescence detection in fractionated methanol extracts of a diesel particulate SRM 1650 subjected to chemical reduction. Detector exposure time: 20 s, unspiked extract; 10 s, extract spiked with 100-fold diluted SRM 1587; 2 s, extract spiked with 10-fold diluted SRM1587. Excitation at 364 nm, n-octane, T = 25 K. Reproduced from [51], with permission.

18.3.4.2 In-ring nitrogen-substituted PAHs

As holds for amino- and nitro-PAHs, also azaarenes and carbazoles, the nitrogen-containing in-ring substituted PAHs, are widely spread in the environment concomitantly with the parent analogues. They originate from geological sources and from anthropogenic emission related to the discharge of industrial effluents and the incomplete combustion of organic material. Many azaarenes and carbazoles are known to induce mutagenic and carcinogenic effects, in particular the benzenoid derivatives of acridines. Furthermore, structurally similar compounds exhibit different toxicities. Hence, there is an obvious need for analytical techniques, capable to discriminate between isomeric azaarenes and carbazoles.

Obviously for this purpose, capillary GC techniques (combined with various MS modes, flame ionization or nitrogen-selective detection) are the techniques of choice, though there are still some difficulties in identifying the heavier isomers. Also, LC techniques (with absorption and fluorescence detection) have been applied, but also in that case distinguishing between for instance dibenz[aj]- and dibenz[ai]-acridine is still problematic.

The potential applicability of Shpol'skii spectroscopy, both in the fluorescence and in the phosphorescence mode, for the determination of azaarenes has been shown in the literature. It has been successfully applied by Garrigues and coworkers for the identification of a number of triaromatic azaarenes in crude oils [54]. A recent study of Kozin et al. was confined to the determination of isomeric benzo- and dibenzo-substituted acridines present in complex environmental samples [52].

The Shpol'skii fluorescence spectra of these isomers in n-octane show good isomer specificity over a wide analyte concentration range, indicating fairly good compatibility of

analytes and cryogenic *n*-alkane matrices. It should be noted that additional confirmation can be derived from the Shpol'skii phosphorescence spectra of dibenzacridines. Except for dibenz[*ai*]acridine, excellent quasi-linear phosphorescence spectra could be recorded. Also, the photostability of the azaarenes considered does not give any problem: upon applying extended sample illumination times up to 5 min no relevant decreases in emission intensities were registered. The only negative aspect is the difference in fluorescence quantum yields observed for the compounds concerned: the detection limits varied from 0.01 ng for dibenz[*ac*]- and dibenzo[*ch*]acridine to 0.1 ng for benz[*a*]acridine. The applicability of the technique is illustrated in Fig. 18.22; positive identification was achieved for
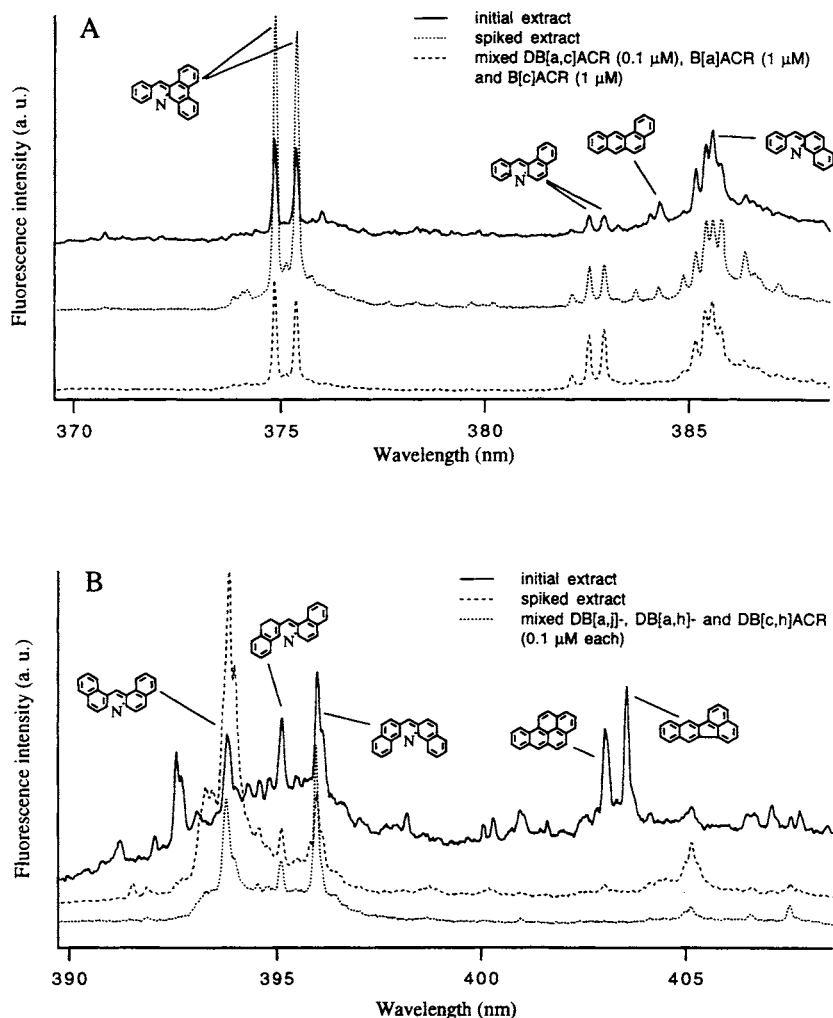
Fig. 18.22. Identification of aza-PACs in Lake Ketelmeer sediment extract by Shpol'skii fluorescence: dibenz[*ac*]acridine, benz[*a*]- and benzo[*c*]acridine upon lamp excitation at 285 nm (A), dibenz[*aj*]-, dibenz[*ah*]-, and dibenzo[*ch*]acridine upon lamp excitation at 303 nm (B). *n*-Octane, *T* = 32 K. Reproduced from [52], with permission.

benz[*a*]-, and benzo[*c*]acridine as well as for dibenz[*ac*]-, dibenz[*aj*]-, dibenz[*ah*]- and dibenzo[*ch*]acridine. As expected, the above mentioned dibenzacridine isomers could be detected by phosphorescence as well.

In conclusion it can be stated that Shpol'skii fluorimetry and phosphorimetry is fully applicable to the isomer-specific detection of azaarenes. In comparison to nitro- and amino-PAHs these compounds are far easier to handle.

18.4 CONCLUSIONS

Shpol'skii spectroscopy of parent PAHs was shown to be applicable to various types of samples of ecotoxicological interest, like sediments or biota extracts. Because of the excellent identification capacities of the technique, it can be used in a qualitative way for the identification of unknown compounds and for the assessment of HPLC peak purity. Carrying out such a procedure once for each type of sample would greatly improve the quality of routine HPLC measurements. Considering the potential of Shpol'skii spectrometry as a quantitative analytical technique, a point questioned in the literature, it has been shown [2] that accuracy and precision of the method are fully adequate if care is taken to assure reproducible sample preparation and cooling procedures, and if a proper internal standard is employed. The application of Shpol'skii spectrometry as an extra independent analytical technique will be especially useful in case large discrepancies are observed between analytical results and references values or between analytical results obtained with different (chromatographic) methods.

Conventional Shpol'skii spectrometry can also be employed to the biological monitoring of PAH exposure (accumulation of parent PAHs), using an extraction and cleanup procedure equal to that routinely applied for HPLC analysis. It was demonstrated that Shpol'skii analysis in crude, lipid-rich extracts is also possible, but matrix distortions have to be accounted for [21]. For such complex analytical problems, laser-excited Shpol'skii spectrometry (LESS) is particularly useful. Because of their relative polarity, PAH metabolites and amino-derivatives are not fully compatible with the matrix. However, the sensitivity can be greatly enhanced for phenolic hydroxy-metabolites if chemical derivatization is applied. Utilizing enzymatic hydrolysis, derivatization, and LESS detection, monohydroxy metabolites of benzo[*a*]pyrene can be determined at sub-ppb levels in the bile of fish.

Obviously, Shpol'skii spectrometry (and especially LESS) is not suitable as a rapid, low-cost, screening technique. It requires sophisticated, advanced instrumentation and cryogenic temperatures. An interesting result, however, is that the concentration of 3-OH-BaP in fish bile was found to be correlated with 1-OH-pyrene; the latter is easily determined with more conventional techniques, since its concentration is much higher. Synchronous fluorescence spectrometry (SFS) was found to be a very rapid and practical method; the sensitivity for this marker metabolite is sufficient for most field applications.

To date, contrary to the determination of PAHs, which is routinely carried out in numerous laboratories, the analysis of nitrogen-PAHs or PAH metabolites has received minor attention. It should be realized that the amount of metabolites excreted is a direct measure for the integrated uptake, and that combining PAH metabolite levels with environmental levels of parent PAHs provides information on bioavailability. Nitrogen-substi-

tuted PAHs are of particular importance from an environmental toxicology point of view. Evidently, the fluorescence techniques discussed in this chapter can play a significant role in this field of research.

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Chapter 19

Applications of liquid chromatography-mass spectrometry in environmental chemistry: characterization and determination of surfactants and their metabolites in water samples by modern mass spectrometric techniques

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CONTENTS

19.1 INTRODUCTION

Worldwide, the production of surface-active compounds, so-called surfactants (includ-ing soaps), had a volume of nearly 30 million tons in 1996 [1]. Surfactants represent one of the major and most multi-purpose groups of organic compounds produced and therefore belong to the organic chemicals with the highest production rate. Besides soaps with a share part of 9 million tons, synthetic surfactants are applied as powders and liquids as

well as soap and syndet (synthetic detergents) bars. The main fields of use are industry (cleaning products, food and industrial processing), household (laundry, dishwashing, etc.) and personal care (soaps, shampoos, cosmetics). The worldwide consumption of synthetic surfactants can be specified as 2.8 million tons of linear alkylbenzene sulfonate (LAS) as the main anionic surfactant, 2.5 million tons of alcohol derivatives as non-ionic surfactants and 2.1 million tons of other surfactants – cationics and amphoterics [1].

Since they are used in aqueous systems as surface active compounds, the total quantity of surfactants produced should get into the waste water. According to legislative prescriptions these anthropogenic compounds have to be biodegradable, however, they are not completely mineralized in common biological waste water treatment. This is a consequence of short retention times in biological waste water treatment. Moreover, they are hardly adsorbed by the bacteria because of their hydrophilic character. Therefore non-eliminated surfactants are discharged with the waste water into rivers together with their primary degradation products (metabolites). These are biochemically degraded surfactants: their structure has marginally changed and they should have lost their surface activity. The main problem associated in the past with the presence of surfactants as well as their metabolites was foaming in waste water treatment plants and in the rivers serving as receiving water. Today this effect normally is no longer observed because the poorly biodegradable compounds such as branched alkylbenzene sulfonates (ABS) have been replaced by compounds like linear alkylbenzene sulfonates (LAS), secondary alkane sulfonates (SAS), coconut diethanolamide or alkylpolyglucosides (APG), which are more easily degradable, resulting in primary degradation products, or can even be completely degraded (mineralized) in the waste water treatment plant (WWTP).

Some of these compounds, even in only slightly raised concentrations [2–4], or their metabolites, i.e. alkylphenols (AP) or alkylphenol carboxylic acid, may have toxic effects on the biocoenosis in the biological waste water treatment process. These compounds arising under aerobic and anaerobic biodegradation conditions [5,6], are not only toxic for the waste water biocoenosis but also supposed to be effective as endocrine disrupters, generating estrogenic effects in fish [7–10]).

If the excess sewage sludge is used as fertilizer in agriculture, non-degraded surfactants adsorbed by the sludge in case of rain are remobilized, elute from the sludge and get into the soil. Here they may be further degraded or transported into ground or surface waters. When passing through the soil, they may remobilize organic pollutants which had been adsorbed before on soil particles. Due to their surface activity pollutants such as PAH (polycyclic aromatic hydrocarbons), PCB (polychlorinated biphenyls) are solubilized and may be transported by the surfactants enclosed into so-called micelles [11,12]. Surfactants as particularly persistent anthropogenic compounds [13–21] and their metabolites, e.g. alkylphenol [22], may even get into the drinking water produced from ground and surface water.

In the aquatic environment the presence of surfactants or their by-products can be used as a potential marker of ground water pollution (e.g. ABS) or as an indicator of the ground water's age [23]. Because of their persistence some of them, e.g. linear alkylbenzenes, their sulfonates (LAS) and nonylphenol, may act [24–26] as tracers of domestic wastes in the marine environment and trialkylamines as indicators of urban sewage in sludges, costal waters and sediments [27].

The analysis of surfactants in water is difficult because their molecules are non-volatile,

highly polar and therefore water-soluble. Detection, identification and quantification of these compounds in aqueous solutions face the analyst with considerable problems. Even today, the common techniques for measuring surfactants are spectrophotometric and titrimetric methods, i.e. sum parameter analysis, which provide no information on the individual surfactant. They are insensitive with relatively high detection limits, non-specific and liable to interferences arising from other compounds of similar structure [28,29]. For example, the methylene blue method, which is traditionally used as indicator of potential anionic surfactants, is affected by other compounds containing an anionic group and a hydrophobic moiety. On the other hand, primary degradation products (metabolites) resulting from marginal modifications in the precursor surfactant molecule are not even detected [30].

The high polarity in combination with high water-solubility is one of the reasons why this substance class is difficult to determine. However, this property is essential for surface activity.

Chemical reactions applied for surfactant synthesis use either raw material from the oil industry, renewable natural compounds or a mixture of both. Since surfactants are commercially produced by means of large-scale chemical processes, a mixture of homologues and isomeric compounds differing in their molecular structure (e.g. non-ionics of the alkylethoxylate type may differ in length of alkyl as well as polyether chain) will be obtained. These raw materials contain unreacted precursor compounds and often dummy compounds to disguise the active ingredients. The easier task of the analytical chemist is quality control in production and trade, whereas the environmental chemist has to analyze the compounds from these mixtures and later on from formulations of these mixtures, which are finely spread in the environment. This analytical approach is a challenge combined with problems which will be discussed later.

Due to this dilution in real environmental samples mentioned before, extraction and concentration of surfactants and their metabolites are essential steps in the determination of traces of surfactants in these samples. This pretreatment can be carried out in very different ways. Most of the common procedures are very well tested [31]. In addition, graphitized carbon black (GCB) may be an adequate method especially for the solid phase extraction (SPE) of metabolites from water samples [32], and accelerated solvent extraction (ASE) was used for sediment samples [33]. Alternatively surfactants in sludge and sediment samples may also be extracted by supercritical fluid extraction (SFE) [34].

Furthermore surfactants are not volatile without decomposition, and therefore chromatographic techniques such as gas chromatographic (GC) analysis require preceding derivatization to form volatile derivatives, with the exception of compounds with lower molecular weight, i.e. short-chain surfactants and their metabolites [21,35–37]. For derivatization a large variety of reagents is used. For example anionic surfactants were either desulfonated or derivatized to form sulfonyl chlorides [38], methylsulfonate esters [39] or trifluoromethane sulfonate derivatives [35]. Non-ionic compounds with a low degree of polyethoxylation ($n \leq 6$) were analyzed by GC-MS, either intact [40] or derivatized (i.e. as trimethylsilylesters for triethoxylated amines [41], or trimethylsilyl- and methylesters for alkylphenol carboxylates) in industrial blends [42] or in environmental samples [13,19,43–48]. Each derivatization step is synonymous with target analysis producing volatile derivatives of the expected compounds. In this way, however, other unknown surfactants which are simultaneously present but differ in structure are discriminated.

Moreover, high temperature GC for derivatives [49] or pyrolysis-GC [50] of compounds was successfully used for non-ionic surfactants.

Liquid chromatography (LC) either using thin-layer (TLC) or high performance column chromatography (HPLC) under normal (NP) or reversed phase conditions (RP) [31,51] with fluorescence (FL) or ultraviolet (UV) detection is widely used for the analysis of anionics and non-ionics in environmental samples if they have a chromophoric group (LAS, APEO) or after derivatization [31,52]. If a chromophore is lacking, an evaporative light-scattering detector (ELS) or refractive index (RI) detector must be applied. Conductivity is used for charged molecules and therefore is used alternatively to the RI detector for anionics and cationics. Besides some other disadvantages, e.g. limitation to isocratic mobile phases using RI detector, the greatest disadvantage of these detector systems is that they only provide a signal, but no substance characterization.

An excellent review on this topic and existing chromatographic alternatives was provided by Schmitt [31], covering the analysis of surfactants by GC and LC techniques. By means of LC on reversed-phase material, surfactants not only in complex matrices but also in industrial blends are often insufficiently separated. Ion-pairing chromatography [53] or addition of buffer [54] improves the separation of anionic surfactants and their sulfophenyl carboxylates (SPC). LC separations of non-ionics with polyethoxylate chains were extensively tested [52]. Alternatively non-ionic surfactants are separated by supercritical fluid chromatography (SFC) much better than by RP-LC if carbon dioxide (CO_2) is used as mobile phase [40,50]. Non-ionic surfactants of the alkylethoxylate type can also be separated by SFC after derivatization with perfluorbenzoylbromide and detected by electron-attachment negative ionization (EANI) [55]. The high separation potential of capillary zone electrophoresis (CZE) was also used for the separation of non-ionic surfactants of alkylethoxylates [56] and alkylpolyglucosides [57] in industrial blends or LAS from other sulfonated compounds [58], resulting in baseline separations within 4 min.

But all these methods applied in combination with GC, LC, SFC or CZE are only more or less specific for the detection of these compounds if unspecific detector systems like FID (flame ionization detection), UV, UV-DAD (diode array detection), FL, RI, LSD or conductivity are applied. Even UV spectra obtained by DAD often give only few information. Furthermore coelution effects can be observed during the LC separation of surfactant-containing extracts resulting in non-reproducible retention behavior [59]. This is recognized, however, only in those cases where substance-specific detection methods such as mass spectrometry (MS) are applied [18].

Mass or tandem mass spectrometry (MS-MS) is more advantageous than the techniques cited before because this method provides the spectroscopist with a lot of additional information, which is missed by unspecific detector systems. According to the ionization method applied, soft or fragment generating ionization, MS provides molecular weight information and distribution or structural information about surfactant oligomers. Additional structural information such as the degree of branching, the isomer distribution and the location of unsaturation and side chains is provided by MS-MS. MS detection without preceding separation (flow injection analysis; FIA) and combined with most different separation, ionization and insertion methods in the examination of surfactants in industrial blends as well as environmental samples has been used for the first time during the 1980s [41,60–64]. The increase in information was considerable. The literature on the detection of surfactants by means of the most common MS methods is referred to in detail, starting

from the beginnings of desorption ionization techniques such as field desorption (FD), fast atom bombardment (FAB), desorption chemical ionization (DCI), laser desorption (LD), electrospray (ESI) up to 1992 [65] and thermospray process (TSP)-MS methods up to 1995 [59].

During recent years reduced quantities of analyte have been necessary for MS investigations because the separation by LC could be improved and in parallel the sensitivity of the mass spectrometers increased. At the same time these developments led to more frequent application of other high resolution separation methods like SFC or CZE which produce only low flow rates. These methods were coupled on-line with MS for the determination of more or less polar, non-volatile compounds in environmental samples. Mainly the interfaces ionizing under atmospheric pressure conditions like electrospray (ESI), ion spray or atmospheric pressure chemical ionization (APCI) as the most actual and modern interface types were used either for on-line LC separations (LC-MS), SFC-MS, CZE-MS or flow injection analysis (FIA-MS) bypassing the analytical column. Combined with a new generation of MS equipment and the interfaces mentioned before, LC-MS as well as FIA-MS meanwhile had not only become the most widespread but also routine methods applied in environmental laboratories. Identification by skimmer or source CID (collisionally induced dissociation) under variation of collision energy or using MS-MS and MS for CID on tandem devices had become much easier with this new equipment, in spite of the fact that daughter ion libraries are not commercially available. The existing laboratory-made libraries [66] are not adaptable to mass spectrometers other than the same type.

Soft ionization with these interfaces take place under atmospheric pressure conditions, therefore atmospheric pressure ionization (API) is used as generic term for these methods. The different interface types, ESI and APCI, ionize polar compounds. ESI, also called ion spray, presents the advantage that especially very polar compounds are softly ionized, whereas the more medium polar compounds are ionized preferentially by APCI without fragmentation; i.e. some compounds are ionized either by ESI or APCI according to the polarity of their molecules. To demonstrate this variability in the ionization potential of the interfaces, Fig. 19.1a,b presents the LC-MS total ion currents of a household detergent containing a mixture of different surfactants which vary in polarity [67]. TIC was recorded by (a) APCI and (b) by ESI, both in the negative mode. Signals, present in (a) are high, dominating the TIC, whereas the same compounds can be observed as small signals in (b) and vice versa. On the one hand the behavior of both API interfaces may be seen as advantageous, because selectivity and sensitivity have increased many times over, compared with TSP ionization covering the larger spectrum of compounds. On the other hand analysis has become more time-consuming because of this selectivity. Compounds of interest contained in the complex mixtures might be discriminated in ionization and therefore cannot be recognized. So it might become a real problem in environmental analysis to fulfil the task of a complete detection of all compounds present. For example, LAS contained in a mixture together with non-ionics can only be detected in the negative ion mode and non-ionics vice versa, whereas TSP(+) ionization is able to detect both – LAS, however, with reduced sensitivity. To overcome these problems by changing from positive to negative ionization in FIA-MS and MS-MS mode, analysis may be quickly done, but in LC it will be a question of time available for analysis.

With both types of interfaces, ESI and APCI, mainly single-charged positive ions [M +
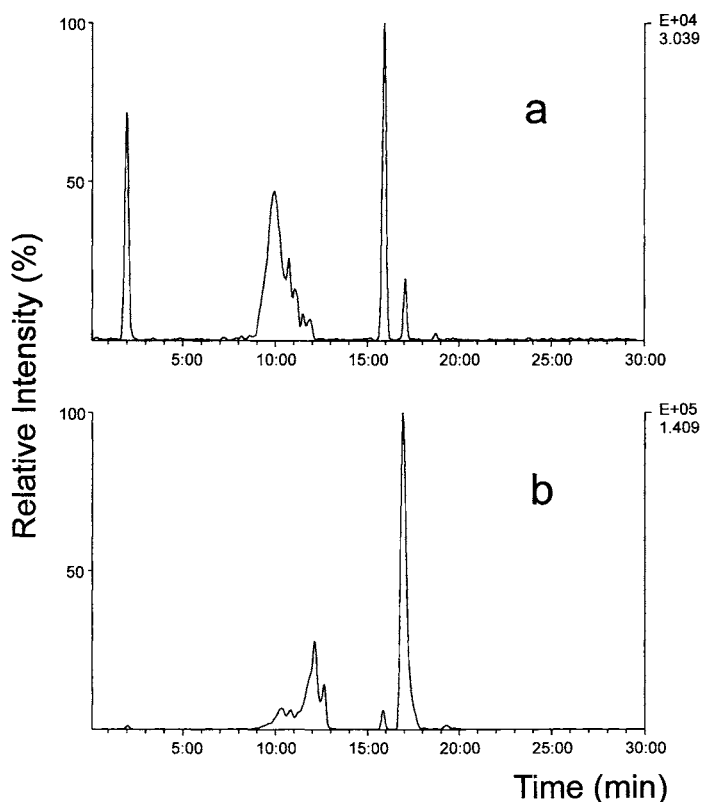
Fig. 19.1. (a) APCI-LC-MS(−) and (b) ESI-LC-MS(−) total ion chromatograms of identical household detergent mixture of different surfactants varying in polarity [67].

H]$^+$ are generated from the molecules in the positive ionization process. If the ionization agent ammonium acetate or for ion-pairing purposes ammonium acetate is added, ammonium adduct ions [M + NH$_4$]$^+$ but hardly any fragments are formed. [M + Na]$^+$ ions as impurities resulting from sodium ions, for example from glass ware, were observed. In accordance with the TSP [59] compounds containing a nitrogen atom in this case were ionized almost exclusively as molecule ions [M + H]$^+$. Ionic nitrogen compounds were ionized as [M]$^+$ ions. Molecular weight information is provided because fragmentation is suppressed. However, multiple- charged ions can also be formed disturbing the information of molecular weight distribution and 'making spectral interpretation difficult' [68]. Increased generation of multiple-charged ions was found as a function of flow rates and molecular composition, i.e. the hydrophobicity of the lipophilic part of the molecules. So the authors found that high flow rates increased the tendency for the formation of multiple-charged ions in the electrospray source (ESI). In addition it could be confirmed comparing alkylethoxylates (stearyl alcohol) with abietic acid ester that the ionization of polyethylene glycol (PEG)-esters more often resulted in multiple-charged ions than PEG-ether compounds. From the results it could be estimated that the presence of unsaturated ring systems seemed to stabilize these ions [68].

If FIA-MS(+) is applied to the samples, the compounds are separated in the spectra according to their different m/z ratios. This helps to identify mainly non-ionic surfactants of the alkyl- and arylpolyether type because of their equidistant signals with $\Delta m/z$ of 44 or 58. This distribution is originating from polyethylene or polypropylene units or a mixture of both glycolether structural elements in their molecules (EO/PO-block polymers).

Negative ESI or APCI ionization in parallel to FAB(−) [65] and TSP(−) [40,59] is very well suited for detection and characterization of compounds which can be easily ionized in the negative mode. In this way predominantly anthropogenic anionic surfactants such as alkylsulfonates and alkylbenzene sulfonates (ABS or LAS), alkylsulfates, alkylphenol-ethercarboxylates and alkylethercarboxylates can be ionized, detected and identified in industrial blends as well as in complex matrices from environmental samples [69]. For other surfactants, i.e. alkylethersulfates (AES), ionization by ESI (+/−) is the only sensitive way for ionization without the loss of the SO_3 moiety. A fission of the bond would pretend non-ionic alkylpolyglycolether ions as observed with APCI(+) [70] and TSP(+) [59]. Moreover, APCI(−) and TSP(−) provide AES ions which are strongly reduced in sensitivity [70]. The biochemical degradation products of non-ionic (APEO) and anionic (LAS) surfactants, predominantly negatively charged ionic compounds (i.e. carboxylates like alkylphenolethoxycarboxylates (APEC), glycoside alkyl carboxylates [71] and sulfo-phenyl carboxylates (SPC) [53,72]), however, can be ionized in the positive as well as in the negative mode. Sensitivity in the negative mode is much higher.

The fact that these soft ionization procedures do not form the structure-characteristic fragments which are essential for identification, but molecular or cluster ions, is often considered to be disadvantageous. This is especially the case when there is no possibility for targeted and reproducible generation of fragment ions due to the equipment configuration. However, the screening on polar, organic compounds by LC-MS is often disturbed by poor LC separations of the different compounds in the samples. The worst that might develop would be that the mixture of molecular and fragment ions arising under these conditions (source CID) would prevent the interpretation of such spectra instead of promoting it. Therefore the molecular weight information as well as the certainty of optimized separation should be the first step when using MS for screening. The second step, even without the option of MS-MS, could be the generation of fragments by variation of the cone voltage of the system (skimmer or source collisionally-induced dissociation (CID)).

However, if the mass spectrometer is equipped for MS-MS, reproducible fragmentation is provided after CID using noble gases like argon or xenon as collision gas and optimizing the collision energy in parallel. Structural information, which is necessary for identification, can be received in this way by CID of the parent ions after normal LC separation or using mixture analysis in FIA mode [72–81]. If FIA-MS-MS is used, time-consuming optimizations of the separations, especially during non-targeted analyses, are not necessary. However, the results should be confirmed by LC separations or application of standards and other verification techniques.

Experiences gained in recent years have repeatedly shown that the substance-group-specific determination methods used up to now are very unspecific and not very sensitive in the determination of surfactants in environmental samples [30]. Moreover, metabolites of the surfactants, so-called primary degradation products with marginal or larger structural modifications, but still surface-active will not be detected under these analytical conditions [30].

In this work a comprehensive survey of the existing experience with the analysis of surfactants in environmental samples by modern mass spectrometric techniques other than FAB [65] or TSP-MS [59] will be given. However, although the number of original and review papers in literature has considerably increased during the past decade, numerous gaps in this field still remain to be filled by ambitious environmental analysts.

19.2 PRETREATMENT AND SEPARATION

19.2.1 Extraction and concentration

Normally an isolation and concentration step precedes the determination of surfactants either by means of FIA or after LC-separation. For water samples, in the past the sublation method [82] or, in rare cases, also liquid-liquid extraction [31] were successfully used. While surfactants from solids in the past and today were preferably extracted with methanol to remove other matrix components (either conventionally in the soxhlet [31] or by means of accelerated solvent extraction [33]), today SPE, applied as cartridges or disks, has replaced the other concentration methods for qualitative and quantitative determination of surfactants in water samples. Solid phase micro extraction (SPME) fibers were also used for the concentration of alkylphenol ethoxylates. For the extraction of this non-ionics Carbowax/template resin and Carbowax/divinylbenzene coatings have proved their usefulness. Standard methods are used only if one insists on their application.

Up to now most different solid phases [31,59,83] such as bonded silica (C1, C8, C18) [63,84,85], GBC [86] and styrene-divinylbenzene resins (XAD) [20,84,87] have been applied. Although their efficiency varied, they were suited for the large number of compounds potentially occurring in water samples. But with some SPE materials (i.e. XAD resins) problems were observed when MS was used for detection. Despite most intensive pre-cleaning of this SPE materials oligomeric compounds eluted from the resins and disturbed MS detection [88].

Strongly polar compounds, e.g. the metabolites of LAS, the SPCs, which cannot be concentrated by means of SPE, can then be detected in the lyophilisate [59] of the waters concentrated by SPE. Some authors use other SPE materials like anion exchanger materials (SAX) [53] or GCB [86] for this purpose. Even methods like steam distillation could be used for the concentration of APEO with short ethoxylate chains from water samples. This concentration technique combined with an extraction step [89] was successfully modified for the APEO concentration step from soils treated with sewage sludge [90].

In the literature mainly such methods are described that serve to detect selected surfactants. It was tried to concentrate certain surfactants, but others were discriminated at the same time, i.e. target analysis was applied. The analytical approach to concentrate the largest possible spectrum of different surfactants on SPE material (preferably C18 or LiChrolut® EN) for screening was also carried out. Far-reaching pre-separation was obtained when the SPE materials were eluted as selectively as possible by solvents and their mixtures with increasing polarity. It was reported in detail on the SPE materials as well as the different eluents [59]. The fractions resulting from this more or less selective pretreatment step can be used without any further clean-up for FIA and LC-MS analyses.

19.2.2 Separation of surfactants

Several fundamentally different methods exist for the analysis of complex mixtures of polar compounds by means of MS. With reference to application in surfactant analysis, two of them, the most important ones, are presented and discussed here:

1. high performance liquid chromatography (HPLC) in combination with MS and MS-MS;
2. mixture analysis (FIA-MS-MS) with a preceding screening step by using flow injection MS (FIA-MS) [59,73,74] in combination with soft ionization techniques.

Both methods have advantages as well as disadvantages. The greatest advantage of FIA compared with liquid column chromatography (HPLC) is the low need for time. Within only 1 or 2 min the information about the molecular weight distribution of the compounds contained in the ionized mixture is received.

The greatest disadvantage of FIA-MS is that in spite of the non-specific information about the molecular weight of compounds ionized no structural information is provided which is essential for identification.

Over the last decade the sensitivity of MS equipment has considerably increased. Using TSP ionization about 1–2 ml/min of the mobile phase mixed with the analyte flew into the mass spectrometer. Today a few μl/min already make a complete FIA or LC analysis possible. The sensitivity of ESI and APCI-MS has tremendously increased and enables today the detection of compounds such as surfactants in surface waters and especially waste waters without any preconcentration. Sometimes these 'high' concentrations of surfactants neither require concentration procedures (sublation, liquid-liquid extraction or SPE) nor LC-MS or LC-MS-MS to monitor and identify the water content. Moreover, FIA-MS screening using soft ionization interfaces prior to any CID procedure provides an overview because of the MS separation procedure based on the different mass/charge ratios (*m/z*) of the molecular or cluster ions. With the help of this very fast screening mode – positive or negative MS in FIA mode bypassing the analytical column – the experienced analyst is able to make statements about the presence of frequently used and therefore most important surfactants. The information in these ESI or APCI FIA-MS overview spectra for a first characterization [69,72,75,79–81] can be taken in parallel to FAB [65] or TSP ionization [59] from:

1. equidistant signals, characteristic for some non-ionic and anionic surfactants;
2. the selectivity of negative ionization for anionic surfactants.

This screening method may provide a lot of information, however, confirmation of the results is essential if any doubt exists about the interpretation [79]. The ESI and APCI-FIA-MS spectra provide the complete and precise pattern of isomers and homologues which helps identification if computerized surfactant analysis can be applied [91].

If surfactant or metabolite concentrations are low, extraction and concentration steps become essential. SPE with its high variety of SP materials available in the meantime had become the method of choice for the analysis of surfactants in water samples in combination with MS detection as mentioned before. The use of SPE with cartridges or disks for concentration, followed by selective elution, leads to very far-reaching pre-separation if eluents with different polarities and their mixtures are applied. While up to some years ago

off-line SPE was still used, on-line SPE in combination with MS sampler systems today is even able to extract water samples, to desorb pollutants from the SPE materials and to generate MS spectra on-line. With increasing sensitivity of the MS equipment, in foreseeable future SPME will be used for this purpose.

In contrast to FIA, liquid column chromatography (LC) is very time-consuming. Separation with normal columns takes between 20 and 60 min or even more. In addition long-lasting equilibration steps between two gradient runs are required. Moreover, extracts on the reversed phase columns (RP) for non-targeted analysis of waste waters, surface waters and even drinking waters may cause problems in LC separation. Because of high concentrations of matrix compounds with surface-activity the separation efficiency of RP-LC columns decreases [18,59,66]. Problems in separation as a result of retention time shifting or coelution effects can only be recognized with the help of substance-specific detectors like MS. Especially selectivity of mass trace analysis and time-consuming column cleaning procedures prior to separation may help to solve this problem and allow the qualitative and quantitative determination of surfactants even in complex matrices. Despite all advantages of FIA-MS and FIA-MS-MS, LC separation is the most important procedure in the analysis of environmental samples because biochemical degradation generates new compounds, and standard material is not available for the anthropogenic part of the water pollution. Mass/charge (m/z) ratios of ions of isomeric and homologue compounds as well as quite different compounds may often be identical if low and medium resolution mass spectmenters are used. If fractionation of the compounds extracted by SPE fails, ionization in the positive as well as in the negative mode is possible besides other MS-MS techniques for differentiation and confirmation [59] and should be used. The same problem with the differentiation of PEG and LAS mentioned in the literature [59] would not arise in FIA-ESI or FIA-APCI-MS application because PEG and LAS can be ionized only alternatively – positive or negative – whereas FIA-TSP(+) is not able to differentiate between PEG [$(HO-(CH_2-CH_2-O)_m-H)NH_4]^+$ ($m = 7$) and linear (LABS) or branched (ABS) alkylbenzenesulfonate [$(C_nH_{2n+1}-C_6H_4-SO_3H)NH_4]^+$ ($n = 12$). Both compounds would be ionized in the positive TSP mode with ions at m/z 344, and differentiation would be impossible [92].

A large number of examples exists for FIA-ESI or FIA-APCI ionization where characterization is impossible and therefore LC separation must precede characterization. Otherwise MS-MS spectra from mixture analysis under this condition will pretend the fragmentation pattern of a single compound, whereas the real daughter spectrum is a mixture of fragments of different compounds with identical m/z ratio [79] (see Section 19.4).

19.3 FIA- AND LC-MS DETECTION OF SURFACTANTS AND THEIR METABOLITES IN STANDARD MIXTURES, INDUSTRIAL BLENDS, SPIKED AND REAL ENVIRONMENTAL SAMPLES

19.3.1 Non-ionics

Compounds of the alkylpolyglycolether (I) ($C_nH_{2n+1}-O(CH_2CH_2O)_mH$) or alkylpoly-propyleneglycolether (II) ($C_nH_{2n+1}-O(CH(CH_3)CH_2O)_mH$) type (Fig. 19.2) as the most common non-ionic surfactants were found by soft ionization MS techniques in great variety of isomeric and homologue compounds in waste waters, surface waters and
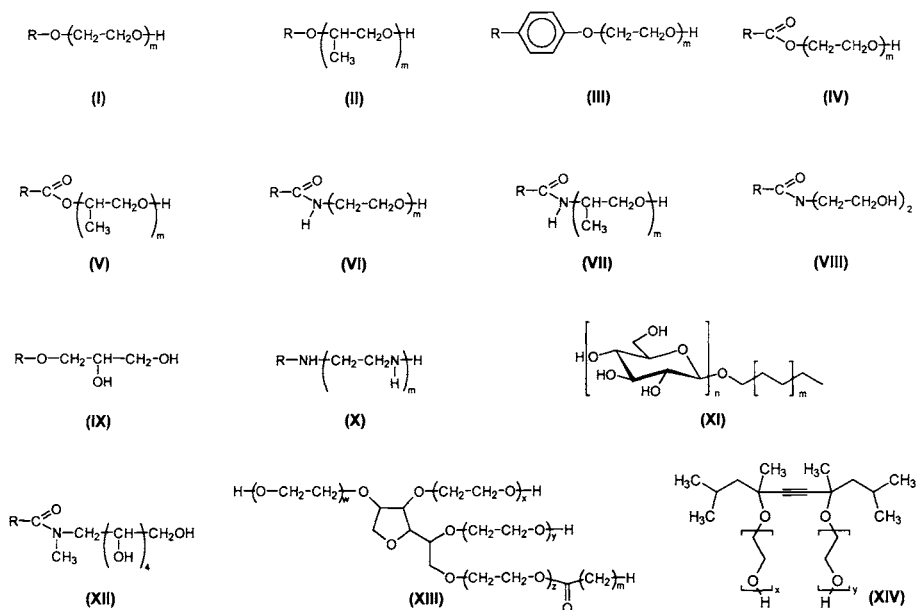
Fig. 19.2. I–XIV. Structures of selected non-ionic surfactants: (I) alkylpolyethyleneglycolethers; (II) alkylpolypropyleneglycolethers; (III) alkylphenolpolyglycolethers (alkylphenolethoxylates or alkyl-arylpolyglycolethers, APEO); (IV) fatty acid polyglycolesters; (V) fatty acid polypropyleneglycol-esters; (VI) fatty acid polyglycolamides; (VII) fatty acid polypropyleneglycolamides; (VIII) fatty acid mono- and diethanol amides; (IX) polyalcohols; (X) polyamines; (XI) alkylglucosides; (XII) alkylglucamides; (XIII) sorbitan esters (Tween®); (XIV) polyethoxylated 2,4,7,9-tetramethyl-5-decyne-4,7-diols (Surfynol®).

even in drinking water [59,65]. The characteristic equidistant signals with $\Delta m/z$ 44 or 58 in their FIA-MS spectra can be used as diagnostic pattern for the screening of non-ionic surfactants of the polyglycolether type. Some more polyether surfactants as shown in Fig. 19.2 also belong to the non-ionic surfactants with characteristic equally-spaced signals. Besides surfactants on polyether basis, however, fatty acid mono- and diethanol amides (Fig. 19.2 VIII), polyalcohols (Fig. 19.2 IX), polyamines (Fig. 19.2 X) alkylglucosides (Fig. 19.2 XI), alkylglucamides (Fig. 19.2 XII), and the sorbitan esters (Fig. 19.2 XIII) (Tween®) belong to this group of compounds, too.

If the characteristic pattern of equal-spaced signals is observed in the MS spectrum, the presence of ionic surfactants which probably have the same structural elements, PEG or polypropyleneglycol (PPG), in the molecule must be taken into consideration. The most important compounds with these characteristics are the alkylethersulfates (C_nH_{2n+1}– ($OCH_2CH_2)_m$–OSO_3H), representing anionic surfactants. Other compounds with the poly-ether structural element in the molecule are alkylethersulfonates (C_nH_{2n+1}–($OCH_2CH_2)_m$–SO_3H), alkylether carboxylic acids (R–(O–CH_2–$CH_2)_m$–CH_2–COOH; R = alkyl; C_nH_{2n+1})–, alkylphenolether carboxylic acids (R = alkylaryl; C_nH_{2n+1}–C_6H_4–) or alkyl-phenolether sulfates, sulfonates and phosphates. All of them belong to the group of anionic surfactants (Fig. 3.18, Section 19.3.2), their presence can be verified by negative ioniza-tion, a more or less characteristic scan mode for most of the anionics.

Positive ionization is the method of choice for the detection of non-ionic surfactants like alkyl- and arylpolyglycolethers generating molecular $[M + H]^+$ or ammonium adduct ions $([M + NH_4]^+)$ in the presence of ammonium acetate. The sensitivity in this mode is very high. Impressions from our results showed a sensitivity which was 10- to 1000-fold higher than in negative ionization.

Different alkyl or polyether chain lengths as well as variation in alkyl chain branching during the large-scale production of alkylpolyglycolethers and adaptation to the special requirements in application may lead to very confusing patterns of signals in the FIA-MS spectra of extracts from industrial and municipal waste water. The behavior, i.e. biodegradability [93] and toxicity [2–4,94] of these compounds in the environment is strongly determined by these special structural elements. The behavior is influenced by characteristic parameters of the surfactant such as linear or strongly branched alkyl chain and the number of ether groups in polyethylene- and polypropyleneglycolethers. The retardation on SPE materials depends on these properties and can therefore be used for appropriate pre-separation of non-ionic surfactants in environmental samples as well as in industrial blends and household detergent formulations.

Standards or industrial blends of non-ionic surfactants were the most frequently examined samples in API-MS determination, covering a large variety of different non-ionics. Reports on real environmental samples, however, are quite rare, and despite their environmental relevance alkylphenolethoxylates (APEO) are found very often. Research work on this topic dominates literature.

19.3.1.1 Alkylpolyglycolethers

A non-ionic surfactant (Brij 35) of the alkylpolyethoxylate type with the general formula C_nH_{2n+1}–$O(CH_2CH_2O)_mH$ used for biochemistry research were analyzed by matrix-assisted laser desorption ionization (MALDI)-MS to compare the separation results of thin-layer (TLC) and reversed-phase chromatography (RP-LC) for these surfactant and to detect impurities within the product [95]. The non-ionic surfactant Brij 35, a mixture of C_{12} and C_{14} homologues ($m = 15$–39), was detected qualitatively as $[M + Na]^+$ and $[M + K]^+$ ions and quantitatively after TLC and RP-LC separation.The equal-spaced ions ($\Delta m/z$ 44) of an alkylethoxylate surfactant were detected qualitatively in the FIA-MS mode originating from a single-use syringe filter used for sample filtration. The ESI interface produced $[M + NH_4]^+$ ions. The ion at m/z 528 from the ESI-FIA-MS(+) was submitted to CID and was characterized by ESI-FIA-MS-MS(+) as alkylethoxylate surfactant homologue $C_{10}H_{21}$–$O(CH_2CH_2O)_mH$ ($m = 8$) [96].

The chromatographic behavior of ethoxylated alcohol surfactants (C_nH_{2n+1}–$O(CH_2CH_2O)_mH$; $n = 12, 14, 16, 18$) applied as technical blends was investigated under normal-phase and reversed-phase conditions. Separation was monitored by MS detection using APCI(+) ionization recording the $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ ions [97]. Different materials used as stationary phases and solvents and their mixtures applied as mobile phases were compared. RP chromatography was able to separate according to the alkyl chain lengths whereas the polyether chain length was not determinating for separation. The influence of the different organic solvents in separation was examined in both modes, reversed-phase and normal-phase. On the contrary the normal-phase chromatography allows separation according to polyether chain lengths in the molecules. The

description of the retention factors of oligomeres with bimodal mass distribution was proposed [97].

The non-ionic surfactant mixture (C_nH_{2n+1}–O($CH_2CH_2O)_mH$; primary alcohol ethoxylates; PAE) ($n = 6$ or 8; $m = 9$–14) and the isomeric secondary alcohol ethoxylates (SAE) with the modified formula CH_3–$(CH_2)_n$–$CH(O(CH_2CH_2O)_mH)$–$(CH_2)_x$–CH_3 ($n + x = 9$–11; $m = 9$) were detected qualitatively by ESI-FIA-MS($+$) in industrial blends used in wool scouring [98]. The FIA-MS spectra of the blends proved a Gaussian partition (statistical normal distribution) in molecular weight recognizable by their peak shape. The compounds were ionized not only as [M + H]$^+$ but because of impurities also as [M + Na]$^+$ or [M + K]$^+$ with equidistant signals ($\Delta m/z$ 44). This leads to a confusing diversity of signals because the signals of the surfactant homologues arising from sodium clustering shifted by $\Delta m/z$ 22; potassium clustering led to a shifting of $\Delta m/z$ 38 compared with the [M + H]$^+$ ions. However, the addition of an excess of NaCl given into the solutions suppressed all other cluster ions with the exception of the [M + Na]$^+$ ions resulting in defined molecular weight information. Collisionally activated dissociation (CAD) of these compounds induced by increased skimmer voltages generates fragments. When CAD was applied to the mixture in the FIA-MS mode, a large number of fragment ions like [(HO($CH_2CH_2O)_mH$ + H]$^+$ ($m = 3$–14; $m/z = 151$–635) was found. All ions were originating from the polyether moiety of SAE, whereas no alkyl fragments of the surfactant homologues could be observed [98].

The results of photocatalytic degradation of SAE by UV radiation in combination with TiO_2 particles over a maximum period of 77 h prior to FIA-MS of these mixtures in the positive ionization mode were comparable to those of CAD of untreated compounds. The authors conclude that the carbon–oxygen bond in the SAE molecules can easily be cleaved, resulting in the more stable secondary carbon-centered cation [98].

On the contrary the alkylethoxylates of the PAE type with a distribution of homologues in the FIA-MS($+$) spectra comparable to the SAE ions were fragmented by CAD, resulting in daughter ions which differed from those of SAE surfactants [98].

Standards of non-ionic surfactants of the polyether type were determined using APCI-FIA-MS($+$) for screening purposes. A mixture of alkyl ethoxylates (C_nH_{2n+1}–O($CH_2CH_2O)_mH$) with different alkyl and polyether chain lengths besides a cyclohexane derivative, ethoxylated with a maximum of two ether units were found, all ionized with equidistant signals of their homologues ($\Delta m/z$ 44). Fragmentation resulting in alkyl and polyether fragments took place under these conditions besides ionization as [M + H]$^+$ ions and sodium adduct ions ([M + Na]$^+$) [99]. Qualitative as well as quantitative results from these examinations were used for detection and identification of compounds in tannery waste waters. Non-ionic surfactants of the alkylethoxylate and alkylphenolethoxylate type were determined qualitatively and quantitatively (see Section 19.5.1.1.) besides biochemical oxidation products of polyethylene glycol (see Section 19.5.1.5.)

In the Elbe and Saale rivers, located in the eastern part of Germany, non-ionic surfactants were monitored by FIA-MS($+$) in combination with APCI or ESI interface [88]. Extraction was done by C_{18}-SPE prior to selective elution by diethylether [81]. Ammonium acetate was used in the ionization process resulting in [M + NH$_4$]$^+$ ions. Non-ionics of the alkylethoxylate type with ions at m/z 350–570 ($\Delta m/z$ 44) are to be classified as surfactant of the general formula $C_{13}H_{27}$–O($CH_2CH_2O)_mH$ ($m = 3$–7). The high load of surfactants in this fraction induced coelution of polyethyleneglycol homologues arising as

$[M + NH_4]^+$ ions which are equal-spaced with $\Delta m/z$ 44 at m/z 168–476. The complexity of this overview spectrum gave rise to the assumption that other surface-active compounds were present besides these compounds. To confirm this assumption, mixture analysis by FIA-MS-MS(+) was applied using diagnostic parent scans (see Section 19.4.1.1.) [81].

The non-ionic surfactants with the general formula $C_nH_{2n+1}-O(CH_2CH_2O)_mH$ ($n = 12$, 13, 14, 15, 16 and 18; $m = 1$–20) were detected qualitatively and determined quantitatively (see Section 19.5.1.1.) in samples of raw and treated waste water, river and drinking (tap) water after SPE concentration on GBC material and differential elution by ESI-LC-MS(+) [22]. Methanol/dichloromethane (20/80, v/v) was used for elution. The dried eluates were reconstituted with a methanol/water mixture (70/30, v/v) and ionized in the form of their $[M + Na]^+$ ions after LC separation. The surfactant homologues were separated according to the carbon chain length. The C_{12}-oligomers were identified by the ions at m/z 297–913 ($\Delta m/z$ 44) representing EO homologues with $m = 2$–16 units [22].

Non-ionic surfactants ($C_{11}H_{23}-O(CH_2CH_2O)_mH$; $m = 8$–18) were detected qualitatively as $[M + H]^+$ ions in the influent and effluent of a municipal waste water treatment plant by FIA-ESI-MS(+) using atmospheric pressure ionization (ionspray interface). Equidistant ions with $\Delta m/z$ 44 in the influent extract at m/z 525–965 resulted from C_{11} homologues [75], whereas the ions at m/z 517–869 and 531–883 resulted from $[M + Na]^+$ ions of C_{12} and C_{13} alkylethoxylate homologues [100]. The effluent contained non-degraded alkylethoxylate homologues besides $[M + Na]^+$ ions of polypropylene, recognizable because of their equidistant ions with $\Delta m/z$ 58. For concentration the samples were pretreated by SPE (C_{18}, LiChrolut EN, SAX and graphitized carbon (Envicarb)) or liquid-liquid extraction. The SPE cartridges were eluted with methanol, whereas dichloromethane at pH 2 and pH 12 was applied for the liquid-liquid extraction.

The qualitative and quantitative (comparison Section 19.5.1.) surfactant contents of a WWTP discharge, surface water and foam resulting from an overflow drop were determined in a series of surface water examinations of a tributary of the Elbe river [81]. For analysis APCI-FIA, as well as LC-MS(+/−) was applied using ammonium acetate for ionization support. Besides non-ionic surfactants of alkylethoxylate type ($C_nH_{2n+1}-O(CH_2CH_2O)_mH$) with different alkyl and polyether chain lengths besides other non-ionics like nonylphenolethoxylates and fatty acid polyglycol amines ($R-N^{\oplus}H((CH_2-CH_2-OH)_{x,y})_2X^-$) as cationic surfactants (see Section 19.3.3.3.) were determined qualitatively and quantitatively. LAS as anionic surfactant (see Section 19.5.1.2.) and polyethylene glycol as metabolites of non-ionic surfactants (see Section 19.5.1.5.) were also detected and quantified. For concentration purposes SPE using RP-C_{18} in combination with selective elution was used [40,88]. After a sequential and more or less selective elution [18,59,92] with hexane/ether, ether, methanol/water and methanol the pollutants in the fractions were ionized. The pattern of equal spaced signals in Fig. 19.3a–c provides an excellent overview concerning the more or less selective elution efficiency [40,88]. The alkylethoxylates (●), nonylphenolethoxylates (+) and polyethylene glycol (▲) ionized by FIA-APCI(+) could be observed as $[M + NH_4]^+$ ions, whereas the polyglycol amines (★) appear as $[M + H]^+$ ions. LAS (see Section 19.3.2.1) were ionized in the APCI(−) mode. The hexane/ether fraction contained some of the alkylethoxylate homologues. However, the majority of these compounds was found in the ether fraction besides nonylphenolethoxylates (+) (Fig. 19.3a). A coelution effect, resulting from the high surface activity of the alkyl polyglycol amines, could be observed under methanol/water elution conditions. The
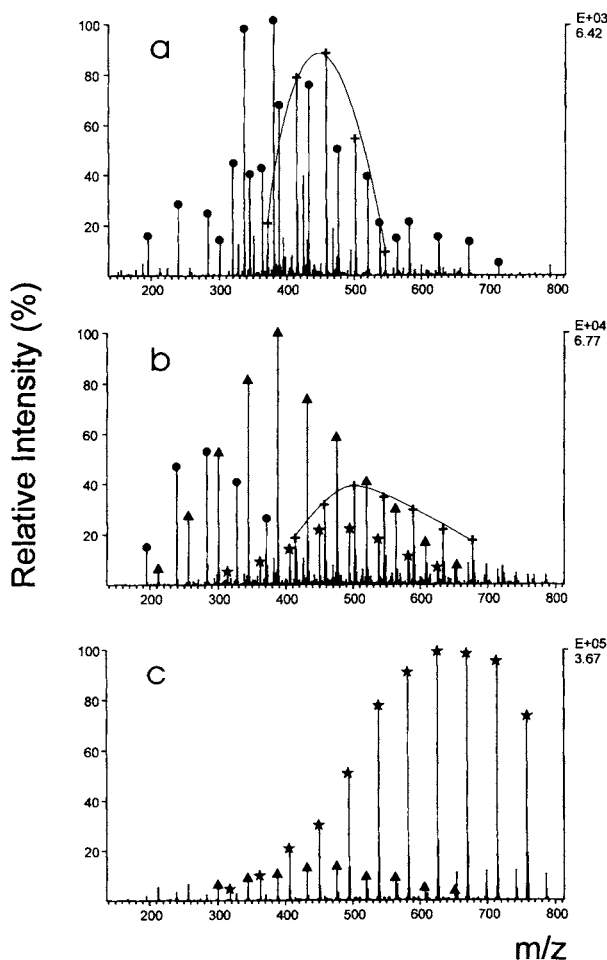
Fig. 19.3. APCI-FIA-MS(+) overview spectra of foam sample from river water concentrated on C_{18}-SPE after selective elution using solvents of different polarities (a) hexane/ether (20/80, v/v), (b) methanol/water (20/80, v/v) and (c) methanol; alkylethoxylates (●), nonylphenolethoxylates (+), polyethylene glycol (▲) polyglycol amines (★) [40,88].

polyglycol amines (★) with short polyglycolether chains could be observed in the FIA-APC-MS(+) spectra of this methanol/water eluate. This fraction contained the polyethylene glycol homologues, too (▲) (Fig. 19.3b), whereas the alkyl polyglycol amines with long ether chains and elevated m/z ratio dominate the methanol fraction (Fig. 19.3c). A small part of PEG could be found in the methanol eluate [40,88].

The ether fraction of the foam sample was examined by APCI-LC-MS(+) [40,88] too. The results of separation can be observed in the total ion current and by UV-DAD trace (220 nm) after LC separation on a RP-C_{18} column (Fig. 19.4). The UV trace and the TIC complement one another. MS active compounds show low UV activity and vice versa.
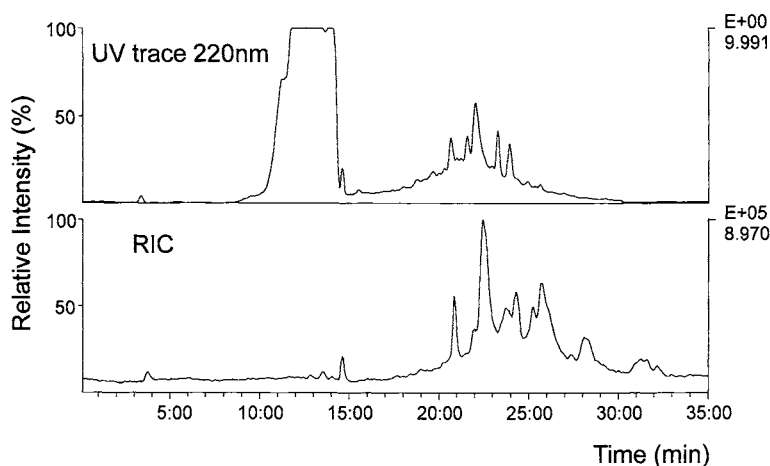
Fig. 19.4. (Bottom) APCI-LC-MS(+) total ion chromatogram and UV-trace 220 nm (top) of foam sample from river water concentrated on C_{18}-SPE after selective elution using ether [40,88].

Substance-characteristic MS-MS-scans in the FIA-MS-MS mode and during LC separation for identification [40,81,88] were also applied to this fraction (see Section 19.4) [40,88].

The WWTP of the city of Thessaloniki, Greece, was investigated for the determination of organic pollutants [79]. For the determination of polar pollutants in influent and effluent extracts APCI and ESI in the positive and negative mode was used in combination with FIA- and LC-MS and -MS-MS. For concentration of the polar waste water content SPE using RP-C_{18} and LiChrolut EN in combination with selective elution was used. After selective elution with solvents or their mixtures with different polarities [18,59,81,92] the pollutants in the fractions were ionized at first for screening purposes by APCI or ESI-FIA-MS(+/−).The results proved that the procedure of selective elution provided fractions ready for MS screening by overview spectra and mixture analysis by FIA-MS-MS for identification.

Applying APCI or ESI-FIA-MS(+) non-ionic surfactants of alkylethoxylate type (C_nH_{2n+1}–O(CH$_2$CH$_2$O)$_m$H) with different alkyl and polyether chain lengths in high concentration and NPEO (see Section 19.3.1.3) were detected qualitatively in the hexane/ether or ether fractions. In the same way polyethylene and polypropylene glycol as metabolites of non-ionic surfactants (see Section 19.3.5.) were detected and carbonylic metabolites of PPG could be detected and identified (see Section 19.4.5.) for the first time, too. Results of FIA-MS screening were checked by LC-MS examinations on RP-C_{18} columns. Mass trace analysis proved that non-ionic surfactants of alkylethoxylate type with different alkyl and polyether chain homologues were present and could be separated according to the lengths of alkyl (Fig. 19.5) and polyether chain (Fig. 19.6) [79].

The comparison of MALDI and ESI-MS spectra of non-ionic surfactant blends of alkylethoxylates and other surfactants like ethoxylated sorbitan oleate operated in the positive ion mode was presented [101]. Both different ionization procedures, which produce [M + Na]$^+$ ion clusters, were very useful for this purpose, but the ESI spectra
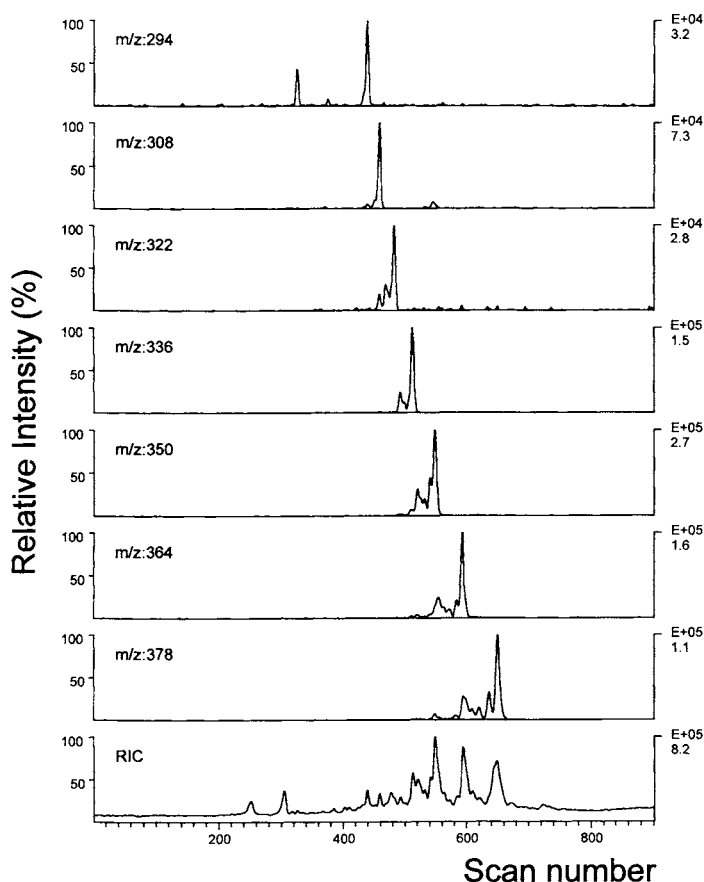
Fig. 19.5. APCI-LC-MS(+) total ion current chromatogram (RIC) and selected mass traces of waste water extract of WWTP Thessaloniki, Greece, presenting separation of non-ionics according to alkyl chain length [79].

generated were more complex and directly related to the molecular structures. MALDI ionization provided simpler spectra which can be interpreted more easily [101].

Laser desorption fourier transform mass spectrometry (FT-MS) in the positive mode was applied for the analysis of different types of non-ionic surfactants. Fatty acid ethoxylates and sorbitan derivatives besides octylphenols (OPEO) with different numbers of glycols, ethylene/propylene oxid blockpolymers with different molecular weights were examined. All non-ionic surfactants with MW <2000 'gave good spectra with little fragmentation showing nice Gaussian peak shapes' [102]. The main disadvantage is fragmentation of high mass surfactants. This will be minimized in future by using MALDI.

The exceptional surface-active effects of fluorine-containing surfactants has made them to excellent auxiliary agents in a large number of industrial processes where non-fluorine containing surfactants must fail. Chemical and physical properties allow the application in combination with acidic, caustic, hot or even highly oxidizing reagents. The properties, behavior under biochemical degradation conditions as well as the extraction, concentra-
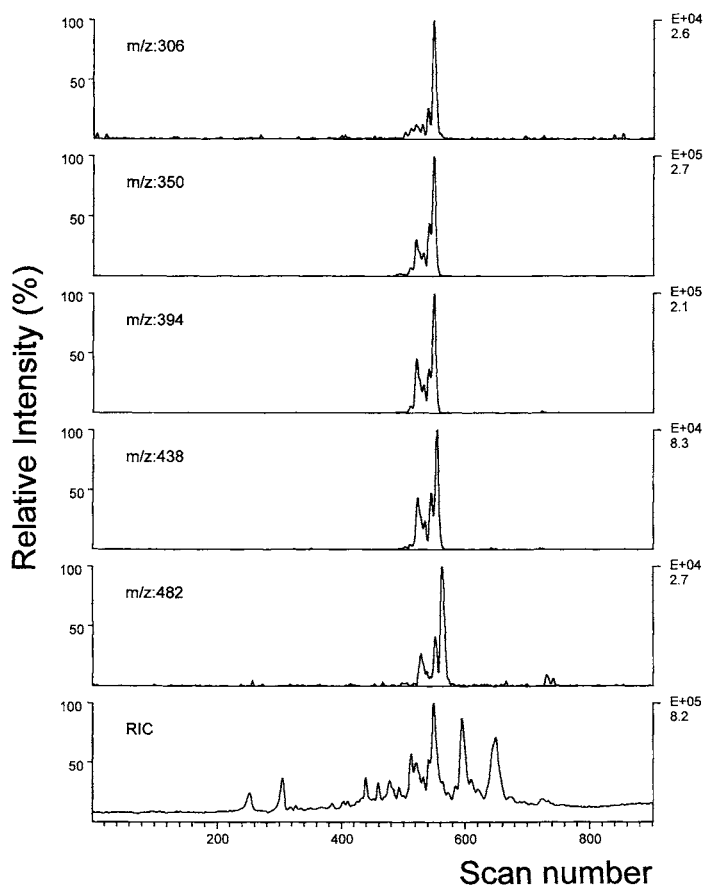
Fig. 19.6. APCI-LC-MS(+) total ion current chromatogram (RIC) and selected mass traces as in Fig. 19.5, presenting separation of non-ionics according to polyether chain length [79].

tion and ionization efficiency of the fluorine-containing surfactants of the general formula $C_nF_{2n+1}-(CH_2-CH_2-O)_m-H$ are described in literature [17]. TSP(+) ionization of the industrial blend Fluowet[®] OTN resulted in three groups of $[M + NH_4]^+$ ions at m/z 470–910 ($n = 6$; $m = 3$–13), 570–966 ($n = 8$; $m = 3$–12) and 670–846 ($n = 10$; $m = 3$–7) according to the alkyl chain lengths. The use of ESI(+) and APCI(+) ionization in the FIA-MS mode resulted in overview spectra more or less different from the TSP spectrum [92]. However, modification of ionization results is observed under variation of extraction voltage in the ionization process. Because of the addition of NH_4 acetate for ionization support, ESI(+) mainly generated $[M + H]^+$ ions at m/z 453–805 ($\Delta m/z$ 44) for the homologues with six carbon atoms in the alkyl chain ($n = 6$; $m = 3$–11) with the dominant signal at m/z 541. APCI-FIA-MS(+) was recorded applying two different source temperatures, 200 and 400°C, which, however, resulted in $[M + NH_4]^+$ ions both with a Gaussian partition of the signals at higher m/z ratios, i.e. with longer polyether chains. In addition the higher source temperature of 400°C produced ions with higher m/z ratios [69]. The finding that APCI ionizes more polar compounds than ESI contradicts own experi-
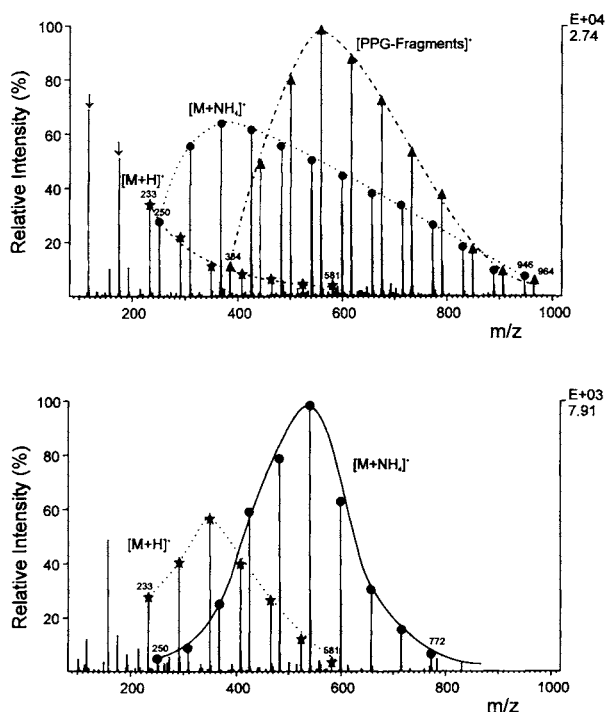
Fig. 19.7. APCI-FIA-MS(+) overview spectra of alkylpolypropyleneglycolether blend ionizied at different source temperatures ((a) 400°C and (b) 200°C) showing $[M + NH_4]^+$ and $[M + H]^+$ ions and $[M + NH_4]^+$ ions of PPG fragments [69].
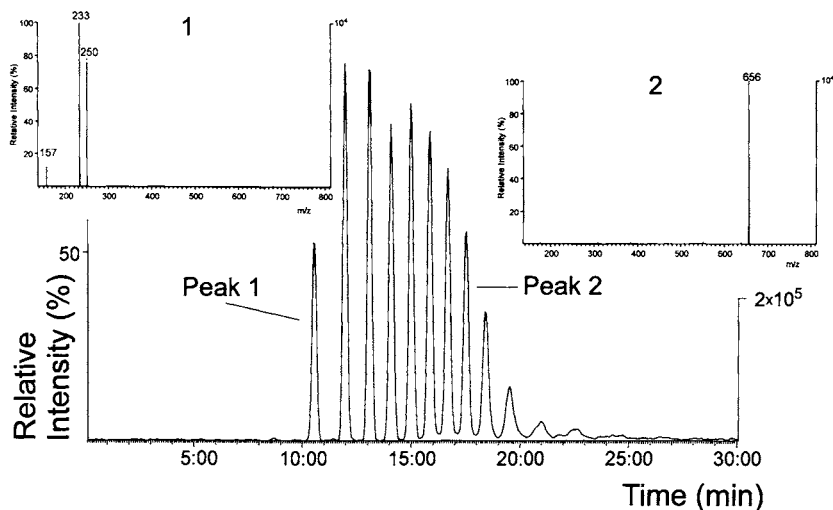
Fig. 19.8. APCI-LC-MS(+) total ion current chromatogram of alkylpolypropyleneglycolether blend as in Fig. 19.7 confirming the separation by mass spectra in the insets (Peak 1, inset 1; peak 2, inset 2) [69].

ences and also those reported by Castillo et al. [99]. Moreover, the generation of ions of non-ionic surfactants with increasing length of the polyether chain and in parallel with increasing temperature could be observed here [69] for the first time. This behavior may be an effect of the electronic partition in this type of molecules because of the fluorine substitution in the alkyl chain.

LC separation by RP-C_{18} resulted in a comparable peak shape as reported for TSP-LC-MS(+) [59,92].

19.3.1.2 Alkylpolypropyleneglycolethers and mixed EO/PO compounds

The non-ionic surfactant mixture with the formula C_nH_{2n+1}–$O(CH(CH_3)CH_2O)_mH$ ($n = 7$; $m = 2$–10) in the form of an industrial blend was examined by ESI and APCI in the FIA- and LC-MS(+) mode. The compound mixture of alkylpolypropyleneglycol-ethers can be concentrated and eluted by C_{18} SPE and selective elution using hexane/ether (1:1) [103]. For ionization support in the FIA-MS mode ammonium acetate was added. The ionization by FIA-ESI(+) was not successful [69]. In contrast to results using TSP(+) ionization [59,92,103] besides $[M + NH_4]^+$ ions (●) with equidistant signals ($\Delta m/z$ 58) at m/z 250–946 $[M + H]^+$ ions (★) at 233-581 and in addition $[M + NH_4]^+$ ions of poly-propylene glycol (▲) at 384–964 ($\Delta m/z$ 58) can be observed by APCI-FIA-MS(+) (see Fig. 19.7a). Moreover, fragment ions of polypropylene glycol (PPG) ($\downarrow$) appeared as dominant ions at 117 and 175 using a standard ion source temperature of 400°C. Reduction of temperature down to 200°C resulted in an overview spectrum more similar to TSP-ionization [69], which now only consisted of $[M + NH_4]^+$ (●) and $[M + H]^+$ ions (★) of the non-ionic surfactant homologues (see Fig. 19.7b). RP-C_{18} LC separation in combination with APCI(+) ionization according to [103] resulted in an excellent separation shown in Fig. 19.8. Insets prove the separation efficiency of this method, showing either the $[M + NH_4]^+$ ion of the non-ionic surfactant C_7H_{15}–$O(CH(CH_3)CH_2O)_mH$ with $m = 2$ at m/z 250 or the homologue with $m = 9$ with m/z 656 [69].

For synthesis of alkylpolyether surfactants monomeric or short chain oligomeric glycol moieties were polymerized. If mixtures of ethylene and propylene glycols were applied for the synthesis, so called EO/PO block polymeres in the form of complex mixtures with the general formula R–O–$(EO)_x$-$(PO)_y$–H will be generated. Because of varying x and y and in addition alkyl chains with different number of carbon atoms polymerized the number of isomers and homologues give rise to the APCI-FIA-MS(+) spectrum presented in Fig. 19.9 [104]. Obviously the identification of these EO/PO-block polymeres can be considered as very difficult, since these compounds cannot be detected by optical systems because of a missing chromophor in the molecules. In parallel these compounds are ecotoxicologically of great interest, because of their persistence against biochemical degradation and toxic effects on water organisms [2,3].

As separation in mass spectrometry applies separation because of different m/z-ratios, isomeric compounds with the identical molar mass cannot be differentiated from one another. Here only separation on analytical columns may help to differentiate between isomers with different EO/PO compositions. The LC separation using RP-C_{18} was successful [104]. As an example of the excellent results in separation only the $[M + NH_4]^+$ mass traces of three pure alternatively EO or PO compounds were plotted together with the total ion mass trace in Fig. 19.10. In all cases presented in the mass traces the alkyl chain
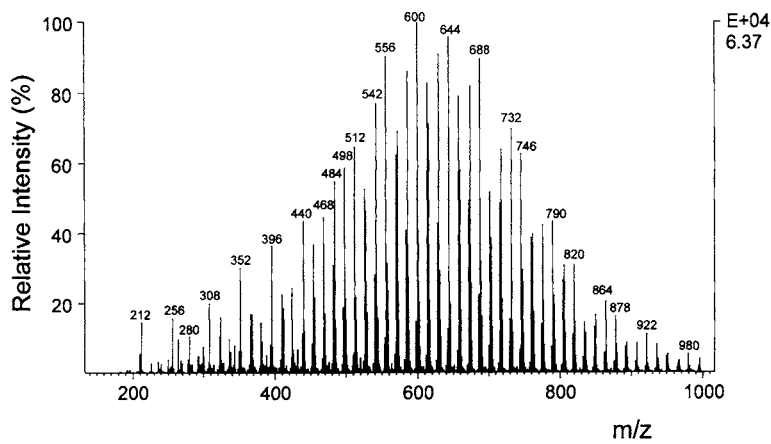
Fig. 19.9. APCI-FIA-MS(+)overview spectrum of fatty acid EO/PO polyglycolether blend ionizied as [M + NH₄]$^+$ ions [104].
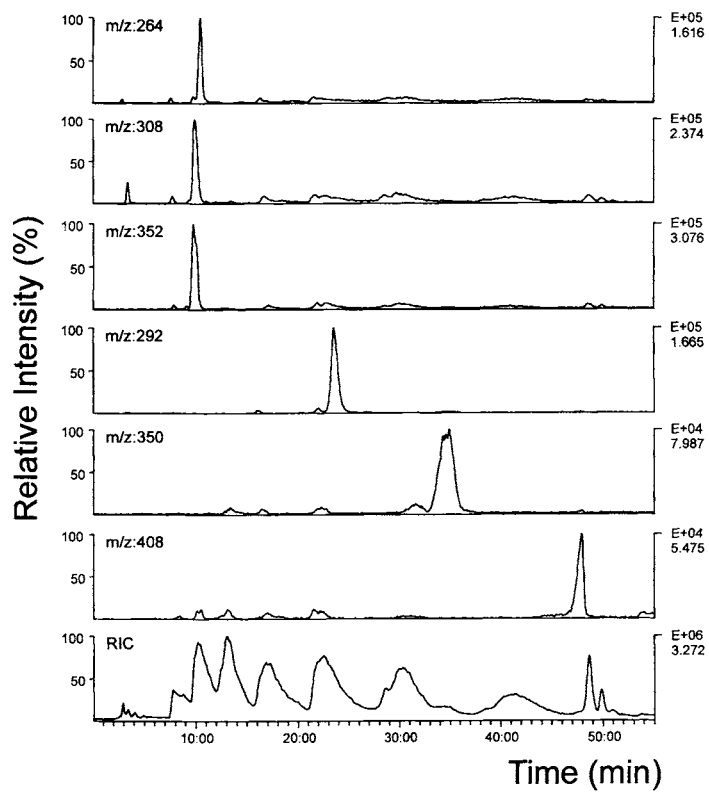
Fig. 19.10. APCI-LC-MS(+) total ion current chromatogram (RIC) and selected mass traces of fatty acid EO/PO polyglycolether blend as in Fig. 19.9, presenting separation of non-ionics according to polyether type [104].

contained ten carbon atoms ($R = C_{10}H_{21}-$). Obviously the LC method applied is able to separate the compounds roughly, as the APCI(+)-TIC proves. Mass trace analysis of R–O–(PO)$_y$–H with m/z 292, 350 and 408 ($y = 2$–4) confirms the separation of compounds containing PO-units, however, the EO-compounds with m/z 264, 308 and 352 ($x = 2$–4) are hidden behind only one signal as reported in literature, too [79].

19.3.1.3 Alkylphenolpolyglycolethers

The octylphenolethoxylates Triton X-100 and X-114 were often used in biochemical research. To compare the separation results of thin-layer (TLC) and reversed-phase chromatography (RP-LC) and to detect impurities within the product matrix-assisted laser desorption/ionization (MALDI) MS was used for purity control [95].

Triton X-100 and X-114 as mixtures of octylphenolethoxylates (C_8H_{17}–C_6H_4–O–(CH_2–CH_2–O)$_m$–H; $m = 3$–18 or 3–13) were ionized in the form of $[M + Na]^+$ and $[M + K]^+$ ions. The mass spectrometric results were in good accordance with the TLC and RP-LC determination of 'number and weight average molecular mass, most probable molecular mass and number of ethoxy groups per molecule' [95]. The disadvantage of MALDI was that MS spectra of the molecules with lower molecular weight especially of the Triton mixture were overlaid by peaks of the matrix applied for an optimized ionization [95].

Triton X-114 'reduced', a mixture of octylcyclohexanolethoxylates (C_8H_{17}–C_6H_{10}–O–(CH_2–CH_2–O)$_m$–H and the precursor compound octylphenolethoxylates (C_8H_{17}–C_6H_4–O–(CH_2–CH_2–O)$_m$–H), was examined. APCI-LC-MS(+) on a RP-C_{18} column was applied to separate this mixture, UV-DAD spectra were recorded in parallel to distinguish the aromatic precursor and the alicyclic reaction product. In Fig. 19.11 the TIC (6) and mass traces of aromatic (2 and 3) and alicyclic (4 and 5) homologues both ionized as $[M + NH_4]^+$ ions equally spaced with $\Delta m/z$ 44 are presented. In parallel the UV trace 220 nm is plotted in Fig. 19.11 (1) confirming the good separation of both compounds [104]. Some more surfactants in small concentration belonging to the non-ionic alkylether type ($\Delta m/z$ 44) could be recognized in the TIC trace.

NPEOs (C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_m$–H; $m = 4$–15) were detected after FIA-APCI-MS(+) qualitatively as $[M + H]^+$ ions at m/z 397–882, as equal-spaced signals with $\Delta m/z$ 44 in a commercial blend. In parallel surface water samples containing agricultural runoff were concentrated by C_8-SPE. For elution of the cartridges methanol/water (95/5; v/v) was used prior to further concentration and FIA-MS(+) determination of the surfactants contained. Besides NPEO with 4–8 glycol units, metabolites, e.g. carboxylates of nonylphenolethoxylate (NPEC) and PEG, were contained in this fraction [105].

The same compound mixture (C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_m$–H; $m = 4$–15) was detected after FIA-ESI-MS(+) qualitatively from an industrial blend used in wool scouring [98]. A statistical normal distribution of the $[M + H]^+$, $[M + Na]^+$ or $[M + K]^+$ ions could be observed with a maximum of the isomer with $m = 9$, represented by the $[M + H]^+$ ion at m/z 617. CAD of this mixture of compounds induced by increased skimmer voltages generated fragments. However, for the generation of daughter ions high voltages (90 V) were necessary. Daughter ions with m/z 177 ($[(CH_2$–CH_2–O)$_4$H]$^+$) and m/z 291 as one of the diagnostic daughter ions of NPEO or APEO respectively [81] (see Fig. 19.12 [106]) appeared under these conditions [98].

The precursor ion scanning of m/z 121 and 133 and multiple reaction monitoring
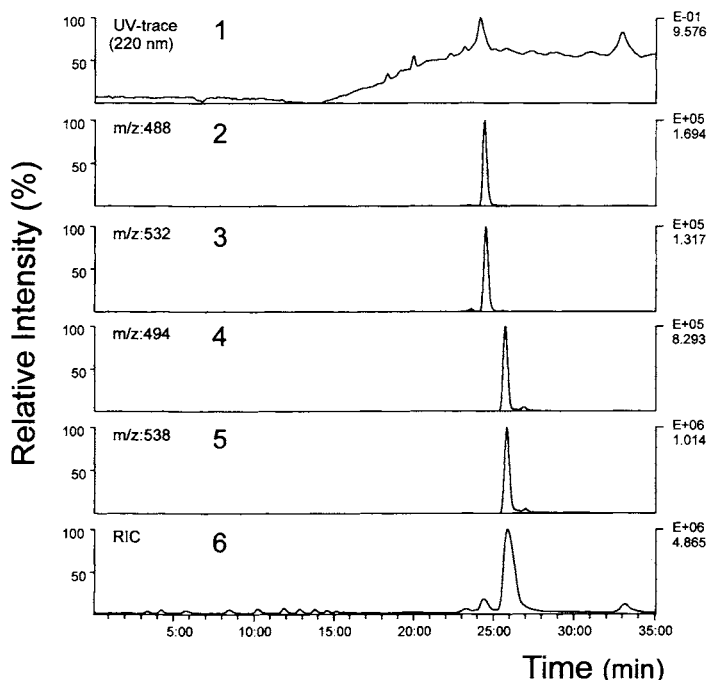
Fig. 19.11. APCI-LC-MS(+) total ion current chromatogram (6) and selected mass traces of (2, 3) of NPEO- and (4, 5) of alicyclic homologue alkylcyclohexanolpolyglycolether and (1) UV trace 220 nm [104].

(MRM) applying API-FIA-MS-MS(+) were used for a rapid screening of nonylphenol-ethoxylates of Igepal CP-720 type in waste waster samples. The parent scan (PS) of 121, characteristic for ethoxylates with 1 to 4 chain units (EO_1–EO_4), and the PS of 133, characteristic for EO_5–EO_{16}, demonstrated a preferential elimination of the EO_5–EO_{16} NPEO. The PS alone was not characteristic for these compounds because linear alcohol ethoxylates resulted in the same precursors; however, monitoring 16 MRM transitions confirmed the results (see Section 19.4) [107].

Since purity of surfactant mixtures is often important for the quality of the product generated with the help of the surfactant mixture, high efforts were made to characterize the mixtures applied. For this purpose API-MS and pyrolysis GC-MS were used for the examination of NPEO homologue mixtures. The mixture showed a distribution of [M + NH_4]$^+$ ions between m/z 264 and 836 equally spaced with $\Delta m/z$ 44. In LC separation three polar components in minor concentration were found by API-LC-MS(+). Results were confirmed by pyrolysis GC-MS [108].

APEOs, i.e. NPEOs or OPEOs (octylphenolethoxylates) (C_nH_{2n+1}–C_6H_4–O–(CH_2–CH_2–O)$_m$–H; $n = 8$ or 9; $m = 3$–10) as industrial blend and standard (Triton 100) respectively were separated together with LAS on a C_1–RP column [109]. Separation was very successful showing the different EO homologues in the LC chromatogram with fluorescence detection. Using APCI-LC-MS(+), however, the total ion current (TIC) chromato-
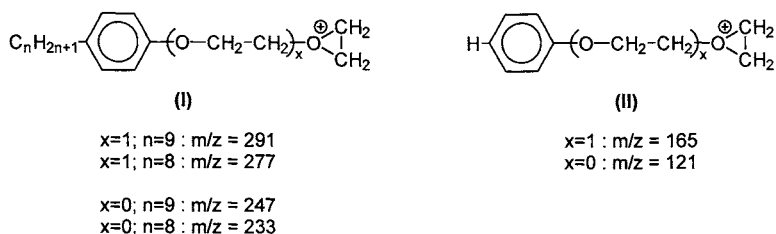
Fig. 19.12. Substance specific fragments for characterization of APEOs [98].

grams were poor in separation. The TIC of these separations was presented together with APCI(+)-spectra of selected signals. The intensive ions which could be observed in the spectra were sodium adduct ions $[M + Na]^+$ (*m/z* 581), $[M + 2Na]^+$ (*m/z* 604) and $[M + 3Na]^+$ (*m/z* 626) of the EO_7 homologue. On the contrary the molecular $[M + H]^+$-ion was small compared to the sodium adduct ions. The compounds had been concentrated prior to separation on C_{18} and SAX SPE cartridges [109]. Samples from river water were handled in the same way, and the concentration of APEO was quantified using fluorescence detection.

Standards of NPEO mixtures with different polyether chains and the reduced homologue, a cyclohexane derivative, ethoxylated with a maximum of 2 ether units, all ionized with equidistant signals of their homologues ($\Delta m/z$ 44) were determined using APCI-FIA-MS(+) for screening purposes [99]. Qualitative as well as quantitative results from these examinations were used for detection and identification of compounds in tannery waste waters (see Section 19.5.1.1) besides biochemical oxidation products of polyethylene glycol (see Section 19.5.1.5)

OPEOs were determined in river waters and effluents of waste water treatment plants in Japan [110]. Besides UV detection APCI- and ESI-FIA-MS(+) was used for detection after clean up to remove ionic surfactants from the methanol eluate of XAD-16 resin. APCI-FIA-MS ionization was promoted by applying ammonium acetate resulting in equal spaced ($\Delta m/z$ 44) $[M + NH_4]^+$ ions starting at *m/z* 312 and ending at 972. LC-separation was successful using a carbon column and ethanol as mobile phase, with the result that a separation according to the ether chain length took place. The partition followed a Gaussian curve with a maximum at $m = 9$ as quantitative APCI measurement proved (cf. Section 19.5.1.1). ESI and APCI-MS-MS was used for identification (cf. Section 19.4.1.3) showing the diagnostic fragments of APEOs – polyether chain fragments and fragment at *m/z* 277 (cf. Fig. 19.12) [110].

APEO, e.g. NPEOs (C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–O$)_m$–H; $m = 1$–20)), were detected qualitatively and determined quantitatively (see Section 19.5.1.1) in samples of raw and treated waste water, river and drinking (tap) water after SPE concentration on GBC material and differential elution by ESI-LC-MS(+) [22]. Methanol/dichloromethane (20/80, v/v) was used for elution. The dried eluates were reconstituted with a methanol/water mixture (70/30, v/v). After LC separation the APEOs were ionized and detected in the form of their $[M + Na]^+$ ions.

In the Elbe river and one of its tributaries, the Saale river, non-ionic surfactants of APEO type were monitored by FIA-MS(+) in combination with APCI or ESI interface

[88]. Extraction was done by C_{18}-SPE. A high load of surfactants in fractions after selective elution induced coelution of all types of surfactants present in these surface waters. The complexity of FIA-MS(+) overview spectra gave rise to the assumption that other surface-active compounds were present besides APEO compounds. To confirm this assumption, mixture analysis by FIA-MS-MS(+) was applied using the diagnostic parent scans (PIS). Using PIS m/z 277 and 291 for the detection and identification of APEOs, NPEOs were confirmed in this way (see Section 19.4.1.3) [81].

Surfactants in a WWTP discharge, in surface water and foam resulting from an overflow drop were determined qualitatively and quantitatively in a series of surface water examinations of a tributary of the Elbe river [81]. FIA- as well as LC-APCI(+) was applied using ammonium acetate for ionization support. NPEOs (C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_m$–H) besides alkylpolyglycolethers and fatty acid polyglycol amines (see Section 19.3.3.3) were found. After a sequential selective elution [18,59,92] with hexane/ether, ether, methanol/water and methanol the pollutants in the fractions were ionized. The patterns of equal-spaced signals were presented in Fig. 19.3a–c. Under the conditions applied alkylethoxylates (●), NPEOs (+) and PEG as metabolites (▲)could be observed as [M + NH$_4$]$^+$ ions after FIA-APCI(+). Polyglycol amines (★) appear as [M + H]$^+$ ions [40,88].

In the course of surfactant monitoring in the influent and effluent of the WWTP of the city of Thessaloniki, Greece, using APCI and ESI in the positive and negative mode in combination with FIA- and LC-MS and -MS-MS NPEOs were found. For concentration RP-$_{C18}$ and LiChrolut EN in combination with selective elution was used. NPEOs besides PPG (polypropylene glycol) and carbonylic metabolites of PPG could be detected and identified for the first time in the hexane/ether or ether fractions (see Section 19.4.5).

Results of FIA-MS screening were checked by LC-MS examinations on RP-C_{18} colums.
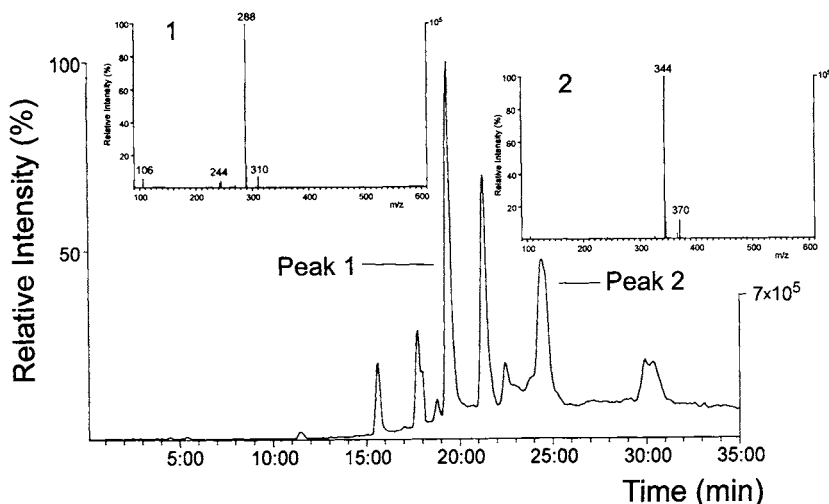
Fig. 19.13. ESI-LC-MS(+) total ion current chromatogram of fatty acid diethanolamide blend (C_nH_{2n+1}–C(O)N(CH$_2$–CH$_2$–OH)$_2$) confirming the separation by mass spectra in the insets ($n = 11$: Peak 1, inset 1; $n = 13$: peak 2, inset 2) [69].

Mass trace analysis proved that non-ionic surfactants of NPEO type with different with varying polyether chain lengths were present [79].

19.3.1.4 Fatty acid polyglycol esters

For the detection of non-ionic surfactants unspecific detector systems like evaporative light-scattering (LSD) or refractive index detector (RI) can be applied, however, they are unable to characterize peaks after LC separation. As UV or fluorescence detection needs a chromophoric group in the molecules, two different introduction techniques with MS detection were applied. ESI-LC-MS(+) as well as ESI-FIA-MS(+) [111] were used for the investigation of polyetoxylated fatty acid esters and the impurities originating from the producing process. These non-ionic surfactants with the general formula ($R–C(O)O(CH_2–CH_2–O)_mH$) were ionized resulting in equal-spaced signals with $\Delta m/z$ 44 because of the polyether units. Both techniques provide detailed characterization of the surfactant blends [111]. However, using both techniques, FIA and LC-MS, polyethylene glycol (PEG) ($HO(CH_2CH_2O)_mH$) could be found in varying quantities besides these surfactants in the blends. LC provided a means of solving the problems with interfering molecular ions, PEG and non-ionic surfactants, observed by FIA-MS. Moreover, dependent on the application of the blends PEG may cause various problems [111].

19.3.1.5 Fatty acid and unsaturated fatty acid diethanolamides

Fatty acid diethanolamides ($C_nH_{2n+1}–C(O)N(CH_2–CH_2–OH)_2$), known as easily degradable compounds in the biological sewage treatment process [59], were ionized by APCI-FIA-MS(+) and ESI-FIA-MS(+) from an industrial blend. As nitrogen-containing compounds the ions of the homologues were generated by both ionization techniques with identical peak shapes of homologues in the form of their $[M + H]^+$ ions at m/z 232 ($n = 7$), 260 ($n = 9$), 288 ($n = 11$) and 316 ($n = 13$) [69]. The LC separation of this mixture of homologues on an RP-C_{18} column is shown in Fig. 19.13 recorded in the ESI-LC-MS(+) mode. The separation efficiency was checked and is demonstrated by the mass spectra as insets [69]. Extraction and concentration of this mixture from environmental samples is possible by C_{18}-SPE with methanol elution after preceding elution with hexane/ether, ether and methanol/water as reported in literature [18].

Unsaturated fatty acids were also used for the synthesis of diethanolamides resulting in compounds with the general formula ($C_nH_{2n-1}–C(O)N(CH_2–CH_2–OH)_2$),. The commercial blend of the oleic acid diethanolamide ($CH_3–C_7H_{14}–CH=CH–C_7H_{14}–C(O)–N(CH_2–CH_2–OH)_2$) could be ionized as $[M + H]^+$ ion at m/z 370 applying ESI-FIA-MS(+). This diethanolamide exhibits only one single ion because of the purity of precursor compounds used for synthesis [104].

19.3.1.6 Alkylpolyglycosides

Alkylpolyglycosides (cf. Fig. 19.2 XI) were examined by ESI- as well as APCI-FIA- and APCI-LC-MS (+/−). These compounds are known as easily degradable surfactants in the biological waste water treatment process [71] and therefore detection in real environmental samples is very difficult. Glucopon®, an alkylpolyglycoside of the general formula
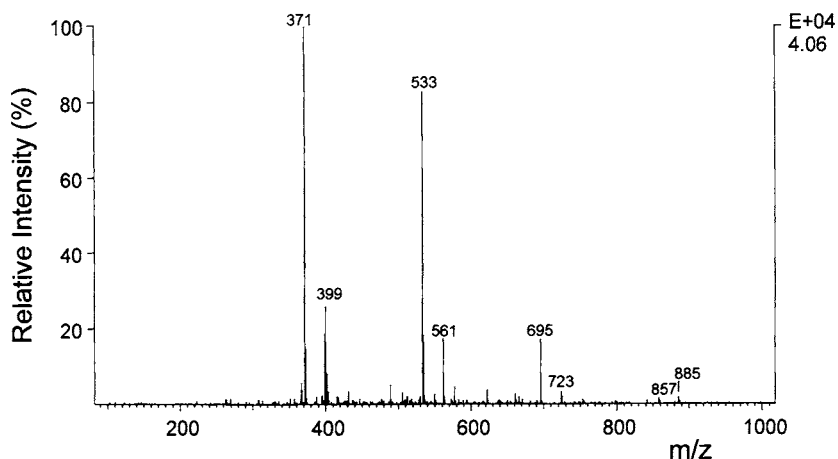
Fig. 19.14. ESI-FIA-MS(+) overview spectrum of alkylpolyglucoside blend presenting [M + Na]$^+$ ions with C_{12}- and C_{14}-carbon chains [69].

$(C_6H_{11}O_6)_x$–R ($x = 1$–3; R $= C_nH_{2n+1}$; $n = 12$ and 14), was used for FIA-screening and LC studies [69]. As reported [59,71] this surfactant mixture can be extracted and concentrated on C_{18} SPE material. Selective elution applying ether can be used for fractionation from matrix compounds prior to MS detection. In contrast to the results reported with TSP ionization, ESI(+) ionization is able to ionize not only the monoglucoside but also the di-, tri- and tetraglucosides in the form of their [M + Na]$^+$ ions with m/z 371, 399 (mono-), 533, 561(di-), 695, 723 (tri-) and 857, 885 (tetraglucoside) (see Fig. 19.14). These compounds only contain alkyl chains with $n = 12$ and 14 carbon atoms. FIA-APCI-MS(+/−) spectra, however, only contain the mono- and diglucoside ions. These ions will be generated by compounds with alkyl chains of $n = 12$ and 14 carbon atoms [69]. Using ammonium acetate for ionization support [M + Na]$^+$ ions were suppressed whereas [M + NH$_4$]$^+$ ions predominantly were generated resulting in ions with $\Delta - 5$ u. Since the identical product and from the same production charge has been used in TSP examinations, the results prove that TSP ionizes the homologues with shorter alkyl chain with higher efficiency than APCI or ESI. These ionization techniques, however, are able to ionize the homologues with more than one glucoside moiety but not with short alkyl chains. This means, the previous assumption received by TSP that the Glucopon® mixture only contained monoglucosides [71] has to be withdrawn now because of the actual results by ESI or APCI.

In parallel to TSP-MS(−) ionization, negative ESI- and APCI-FIA-MS in the presence of ammonium acetate resulted in [M + CH$_3$CO$_2$]$^-$ ions at m/z 407, 435 and 569, 597. The signals at m/z 407 and 569 are formed by the alkylpolyglycoside with the $C_{12}H_{25}$ alkyl chain, while the signals at m/z 435 and 597 belong to the $C_{14}H_{29}$ mono or diglucoside homologues, respectively [69,71]. This shows that positive ESI or APCI ionization was more efficient than negative, but both again were more efficient than TSP ionization.

The LC separation in the APCI-MS(−) mode of a polyglucoside surfactant mixture from another manufacturer (Triton® BG 10) is shown in Fig. 19.15, proving the large number of isomers and homologues present in this industrial blend. The mass traces of the
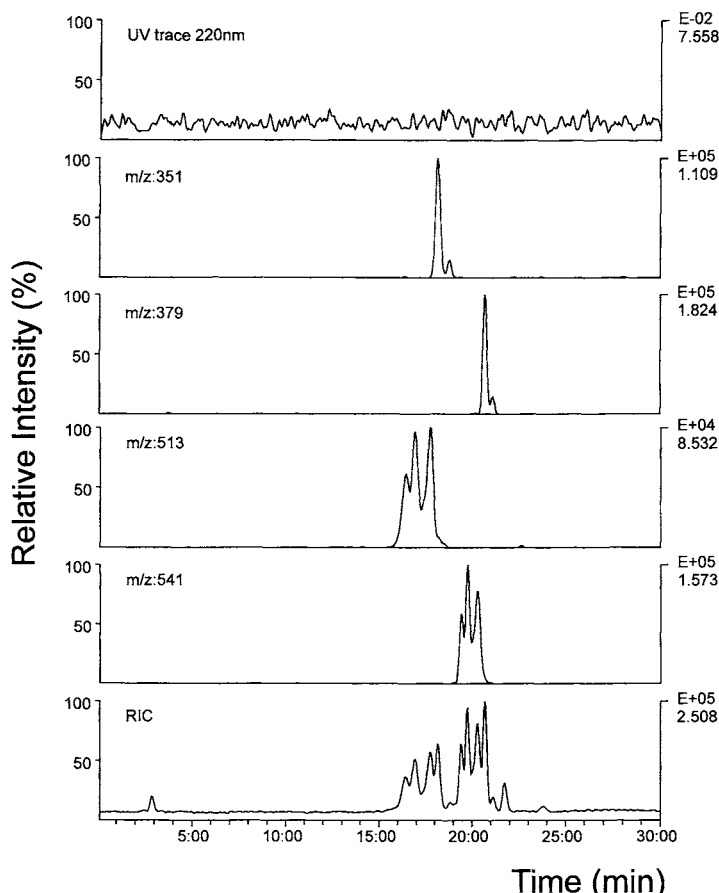
Fig. 19.15. APCI-LC-MS($-$) total ion current chromatogram (RIC) and selected mass traces of alkylpolyglucoside blend (Triton® BG 10) and UV trace 220 nm [69].

dominant [M $-$ H]$^-$ ions of $(C_6H_{11}O_6)_x$–R ($x = 1$; R $= C_nH_{2n+1}$; $n = 8$ or 10) at m/z 351 and 379 and m/z 513 and 541 for the analogous diglucoside ($x = 2$, $n = 8$ or 10)) prove the separation efficiency on a RP-C_{18} column [69].

19.3.1.7 Alkylpolyglucamides

Alkylglucamides belong to the new generation of surfactants produced from renewable natural compounds like alkylglucosides. The general formula of this class of compounds is C_nH_{2n+1}–C(O)N(CH$_3$)CH$_2$–(CH$_2$–OH)$_4$–CH$_2$OH (Fig. 19.2, XII). Since these compounds are produced by the reaction of natural raw materials, a mixture of homologues may result. The industrial blend used for examination contained three homologues because of the number of carbon atoms in the alkyl chain. According to the number of $n = 11$, 12 and 13 carbon atoms the ESI- as well as the APCI-FIA-MS($+$) spectra show three equidistant signals ($\Delta m/z$ 28; –CH$_2$–CH$_2$–) at m/z 378, 406 and 434 (see Fig. 19.16). These peaks are
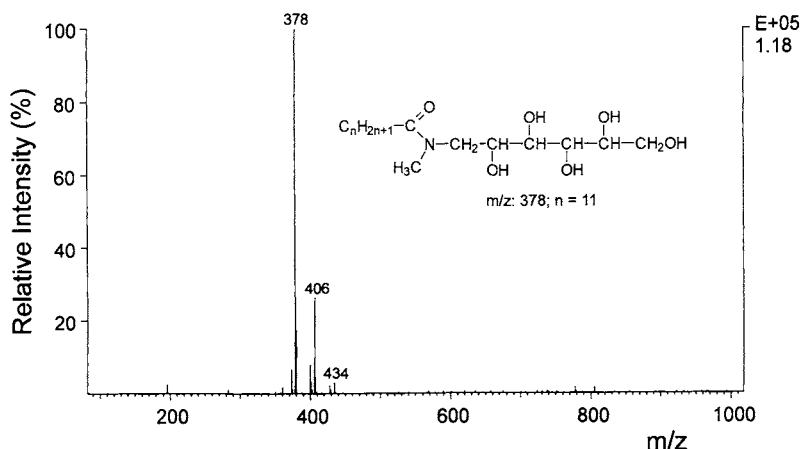
Fig. 19.16.   ESI-FIA-MS(+) overview spectrum of alkylpolyglucamide blend $(C_nH_{2n+1}$–C(O)N(CH$_3$)CH$_2$–(CH$_2$–OH)$_4$–CH$_2$OH) presenting [M + H]$^+$ ions with C_{11}-, C_{13}- and C_{14}-carbon chains [69].

[M + H]$^+$ ions, since most of the N-containing compounds are ionized in the positive mode. In parallel ionization is possible in the negative mode, too, resulting in [M − H]$^-$ ions in the APCI(−) mode or as acetate adduct ions [M + CH$_3$CO$_2$]$^-$ in the ESI(−) mode [69].
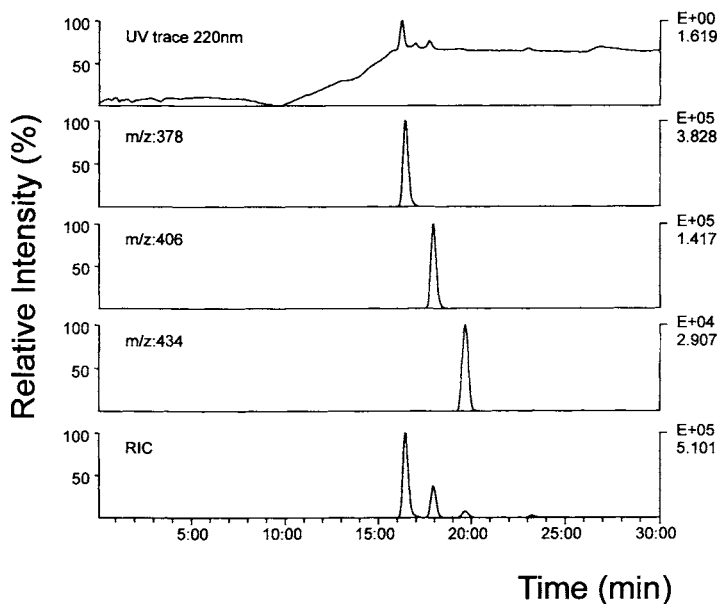
Fig. 19.17.   ESI-LC-MS(+) total ion current chromatogram (RIC) and selected mass traces of alkylpolyglucamide blend as in Fig. 19.16 and UV trace 220 nm [69].

ESI-LC-MS(+) on a RP-C_{18} column led to separation of the homologues according to the length of the alkyl chains as mass trace analysis proved [69] (see Fig. 19.17). UV activity of this compounds was poor, but better than in alkylglucoside detection because of a missing chromophore (cf. Fig. 19.15).

19.3.1.8 Polyethoxylated sorbitan derivatives

Biochemically applied detergents like Tween 20 (ethoxylated sorbitan esters) (cf. Fig. 19.2 XIII) were analyzed by matrix-assisted laser desorption/ionization (MALDI) MS to compare the separation results of thin-layer (TLC) and reversed-phase chromatography (RP-LC) and to detect impurities within the product [95].

Tween 20, the ethoxylated sorbitan carboxylate was ionized resulting in $[M + Na]^+$ and $[M + K]^+$ ions. The number of polyether units in the isomeric and homologue molecules varied, covering the range of 18–34 (CH_2CH_2O) units resulting in $\Delta m/z$ 44 equally spaced signals in the mass spectra [95].

19.3.1.9 Polyethoxylated decyne diols

The different mass spectrometric techniques fast atom borbardment (FAB), time-of-flight secondary ion mass spectrometry (ToF-SIMS), matrix-assisted laser desorption ionization (MALDI), electrospray ionization (ESI) and field desorption (FD) [112,113] were used for generating molecular weight information on a selection of polyethoxylated compounds based on the 2,4,7,9-tetramethyl-5-decyne-4,7-diol (Surfynol®) (see Fig. 19.2 XIV). These compounds were used as non-ionic surfactants with herbicidic effects. The different ionization techniques applied here generate different analytical results according to their varying efficiency to ionize identical compounds in a different manner as also presented in Fig. 19.1 before [67]. The blends Surfynol 420 and 440 produced similar results in their molecular weight (MW) distribution using the different MS techniques, whereas the blends 465 and 485 showed variations. Although the MS techniques applied are providing MW data, little was known about their accuracy [112].

19.3.2 Anionics

The variety of different anionic surfactants produced by the petrochemical industry is larger than the spectrum of non-ionic surfactants. In addition the anionic surfactant mixture 'alkylbenzene sulfonate' is the surfactant with the highest production rate worldwide.

The production spectrum of anionics is reaching from alkyl sulfates, sulfonates and carboxylates to alkylether compounds with an anionic moiety such as alkylethersulfates. In addition arylether derivatives of carboxylates, sulfonates, sulfates and phosphates as well as derivatives of fluorinated compounds belong to this broad spectrum of anionic surfactants. A selection of these compounds with their general structural formula is presented in Fig. 19.18. Only some of them, however, are produced in quantities of $\geq$ 100 000-tons scales per year like the branched or linear alkylbenzene sulfonates (ABS or LAS). The others, e.g. fluorinated compounds, are produced in small quantities, but they
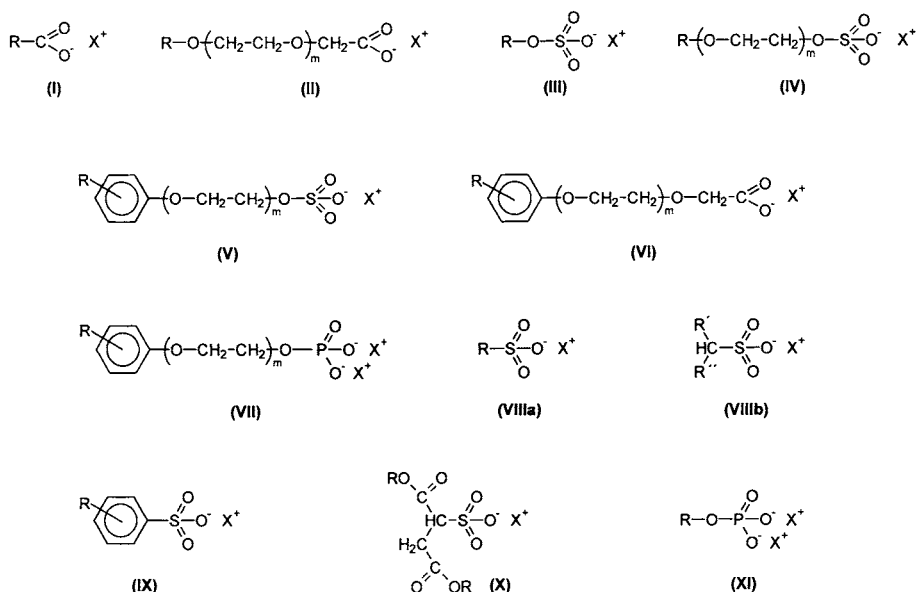
Fig. 19.18. I–XI. Structures of selected anionic surfactants: (I) alkylcarboxylates; (II) alkylpolyethercarboxylates; (III) alkylsulfates; (IV) alkylpolyethersulfates; (V) alkylarylpolyethersulfates; (VI) alkylarylpolyethercarboxylates; (VII) alkylarylpolyetherphosphates; (VIIIa) alkylsulfonates; (VIIIb) sec. alkylsulfonates; (IX) alkylbenzene sulfonates; (X) sulfosuccinates; (XI) alkylphosphates.

can be used for special applications because of their stability against heat, acidic or caustic conditions [17].

For a long time anionic surfactants were determined by the substance-group-specific methylene-blue method [28,29]. However, results of quantitative determination were disturbed by interferences arising from other compounds of similar structure, containing an anionic group or a hydrophobic moiety [28,29,31]. This led to false positive as well as negative results and consequently to alternative determination methods. These photometric methods applied were more or less sensitive and selective compared with methods using separation techniques prior to detection. All together were reviewed in detail by Schmitt [31].

Although this type of surfactants has the largest part in the worldwide total production [1], only few results dealing with the application of API-MS methods for the determination of these compounds were reported in literature. This is in good accordance with the few substance-specific determination methods concerning this topic reviewed some years ago. These former results had been obtained by desorption methods [48,114–124] or in TSP studies [30,66,92,125–129].

As surfactant type with the highest production and application rates anionic surfactants were discharged with the waste waters from households, trade and industry. Surfactants get into the environment mainly via the more or less efficient waste water treatment process. Elimination by adsorptive effects leads to a problem shifting in case sludge is used as fertilizer in agriculture. Primary degradation of the anionics produces metabolites, as observed especially for LAS [130]. Most of these surfactants can easily be eliminated in

the WWTP and therefore can only be found in the influent of biological WWTPs. However, others were not only found in in- and effluents of WWTPs but also in surface waters and even in marine ecosystems [26] together with different types of their biochemical degradation products [53].

Using selective ionization in negative FIA-APCI and -ESI mode for eluates of SPE materials like C_{18}, LiChrolut® EN, GCB or SAX used before for concentration of pollutants in waste water, alkylbenzene sulfonates (ABS or LAS) with the general empirical formula C_nH_{2n+1}–C_6H_4–SO_3H can be observed. If selective elution of the SPE materials C_{18} or LiChrolut EN is applied, these anionic surfactants can be determined in the methanol fraction. The ions at m/z 297, 311, 325 and 339 belong to the LAS homologues with $n = 10$–13 [59,65,131] showing their characteristic pattern of $[M - H]^-$ ions. Ionization in the positive mode as possible with TSP(+) ionization in the form of $[M + NH_4]^+$ ions failed using APCI or ESI [59]. Using negative ionization, nearly all matrix components appearing before with positive ionization now are no longer developed with APCI or ESI(−), and LAS can be observed with an excellent response. The same results and in addition the fragmentation behavior (see Section 19.4.2) were reported from the alkyl naphthalene sulfonates and nitro-substituted benzene sulfonates [131] applying electrospray (ES(−) ionization.

Alkylbenzene sulfonates (ABS or LAS) as the anionic surfactant type with the highest production rate have been studied most frequently by APCI or ESI-MS methods [26,53, 131,132]. This type of surfactant is ubiquitous in waste waters and can even be detected in the marine environment [26,53]. Alkanesulfonates or the more commonly applied secondary alkanesulfonates (SAS) with the general empirical formula CH_3–$(CH_2)_n$–$CH(SO_3H)$–$(CH_2)_x$–CH_3 are detected nearly exclusively in the influents of biological sewage treatment plants because they can be eliminated very easily [59,65]. Therefore their appearance in the effluents of WWTPs is an obvious sign of either an overloading with these compounds or an operating trouble. Information about the MS detection of alkyl ether sulfates (AES) $(C_nH_{2n+1}$–O–$(CH_2$–CH_2–$O)_n$–$SO_3H)$ [70,96,133,134] are quite rare in literature.

The large quantities of linear alkyl benzene sulfonates (LAS) applied all over the world for manifold cleaning purposes and the number of papers reporting MS detection of these compounds in literature is the reason for starting here with papers dealing with these anionic surfactants.

19.3.2.1 Linear alkylbenzene sulfonates

The anionic surfactant dodecylbenzene sulfonate $(C_{12}H_{25}$–C_6H_4–$SO_3^-Na^+)$ was examined besides other anionics by laser desorption fourier transform mass spectrometry (FT-MS) in the negative mode. All anionic surfactants with the exception of laureth sulfate gave one-peak mass spectra originating from the $[M - 1]^-$ ions of the sulfate or sulfonated compounds. Little fragmentation was observed under these conditions [102].

LAS and APEOs together as industrial blend and standard were separated on a RP-C_1 column [109]. Separation was successful showing the different EO homologues in the LC chromatogram with fluorescence detection. Using APCI-LC-MS(+), however, LC chromatograms were poor in separation.

River water (River Rother, South Yorkshire, UK) samples handled in the same way resulted in worse LC-MS chromatograms. Using alternatively positive and negative ioni-

zation for APCI-LC-MS, the ions in the TIC originating from LAS homologues could be easily identified because of the discrimination of APEO under APCI($+$) conditions. The TIC of these separations together with APCI($+/-$)-MS spectra of selected signals were presented. Prior to separation the compounds had been concentrated on C_{18} and SAX SPE cartridges [109].

LAS and their metabolites (see Section 19.3.5.5) were studied at several sampling points in a salt marsh of the Bay of Cadiz by ESI-LC-MS($-$) [53] using RP-C_{18} chromatography. The presence of LAS homologues in decreasing concentration (see Section 19.5.1.2) in relation to the sewage discharge point into the Bay of Cadiz could be determined by LC with fluorescence and MS detection. LAS homologues besides metabolites of LAS (see Section 19.3.5.5) could be confirmed using the characteristic negative fragment ion for LAS and their carboxylic metabolites $CH_3-(CH_2)_n-CH(C_6H_4SO_3H)-(CH_2)_x-COOH$ (sulfophenylcarboxylic acid; SPC) with m/z 183 at elevated extraction voltages. The ESI-LC-MS($-$) mass trace of 183 [53] is presented in Fig. 19.19.

ABS or LAS ($C_nH_{2n+1}-C_6H_4-SO_3H$) could be observed in the influent and effluent extracts of WWTP of the city of Thessaloniki, Greece, by APCI and ESI in the negative mode in combination with FIA- and LC-MS and -MS-MS [135]. The methanol fractions of the C_{18} and LiChrolut EN SPE materials contained these anionic surfactants ionized as $[M - H]^-$ ions at m/z 297, 311, 325 and 339. The FIA-MS($-$) overview spectra of influent and effluent were different because of the pattern of ions. The FIA-MS($-$) influent spectrum contained only these ions, whereas the effluent spectrum contained the same ions and ions with $\Delta m/z \pm 2$ Dalton in addition (see Fig. 19.20). FIA-MS and FIA-MS-MS, however, failed in the determination of the compounds contained in the effluent. All compounds ionized by FIA-MS($-$) in the methanol fraction of the effluent were mainly metabolites of LAS (see Sections 19.3.5.5 and 19.4.5.2) besides small quantities of LAS [135].

The treatment efficiency of a WWTP was checked. Therefore surfactants besides other compounds were determined by screening methods. LAS homologues were detected qualitatively in the influent and effluent of a municipal waste water treatment plant by FIA-ESI-MS($-$) using atmospheric pressure ionization (ionspray interface) as $[M - H]^-$ ions. For concentration of the pollutants the waste waters samples were pretreated by SPE (C_{18}, LiChrolut EN, SAX and graphitized carbon (Envicarb)) or liquid-liquid extraction. The SPE cartridges were eluted with methanol whereas dichloromethane at pH 2 and pH 12 was applied for the liquid-liquid extraction [75].

The LAS homologue ions with m/z 269 to 381 equally spaced with $\Delta m/z$ 14 were generated from the homologues with $n = 8-16$ from the compounds with the general empirical formula $C_nH_{2n+1}-C_6H_4-SO_3H$. Elimination was observed for these compounds as well as for secondary alkane sulfonates (SAS) during the sewage treatment process. SAS with the general formula $CH_3-(CH_2)_n-CH(SO_3H)-(CH_2)_x-CH_3$ ($n + x = 10-15$) were ionized as $[M - H]^-$ ions with m/z ratios 263, 277, 291, 305, 319 and 333 [75].

Surface water from the Saale river, a tributary of the Elbe river [81], was extensively examined. For this purpose effluents of a WWTP, surface water and foam, sampled along the river, were examined qualitatively (see Section 19.3.2) and quantitatively (cf. Section 19.5.1.2) for surfactants and their potential metabolites PEG or PPG (see Section 19.5.1.5). For analysis FIA- as well as ESI-LC-MS($-$) was applied (for concentration on SPE see Section 19.2) [40,81,88]. After selective elution [18,59,92] the pollutants in the
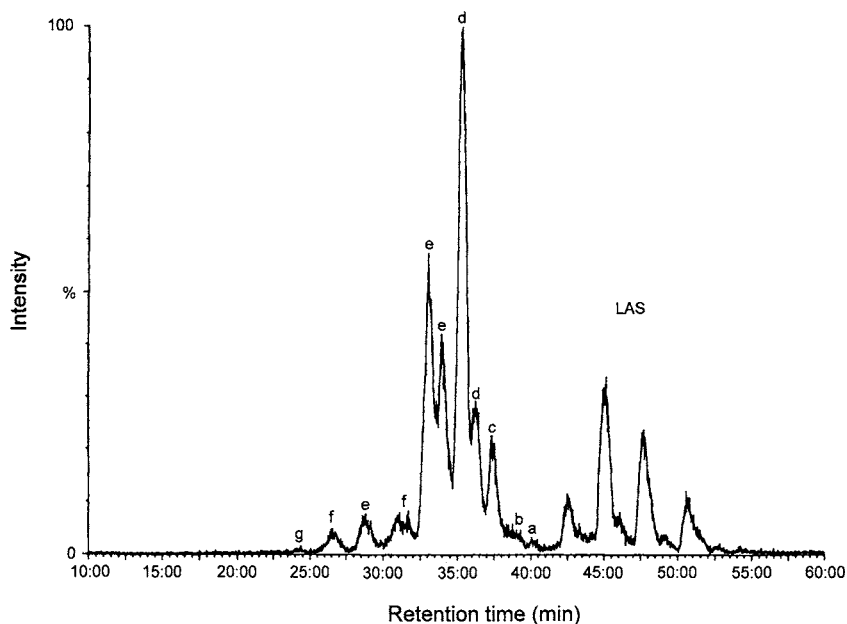
Fig. 19.19. ESI-LC-MS(−)ion current chromatogram (m/z 183) of water sample showing the LC separation of LAS homologues and LAS metabolite homologues. Reproduced with permission from [53]. © 1997 by American Chemical Society.

methanol fractions were ionized in the ESI(−) mode. LAS homologues were ionized in the FIA-ESI-MS(−) mode as $[M - H]^-$ ions at m/z 297, 311, 325 and 339 for the C_{10}–C_{13} compounds.

Methanol fractions of effluent, river water and foam of SPE samples separated on a RP-C_{18} column and detected by ESI-MS(−) under these conditions resulted in TICs
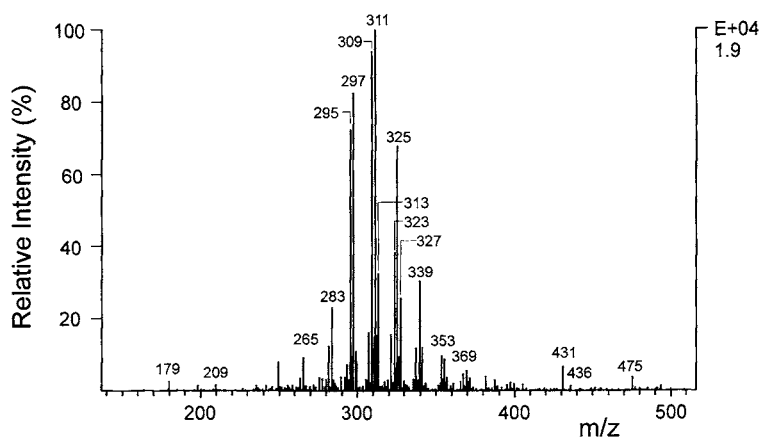
Fig. 19.20. ESI-FIA-MS(−) overview spectrum of C_{18}-SPE waste water extract (methanol eluate) of WWTP Thessaloniki, Greece [135].
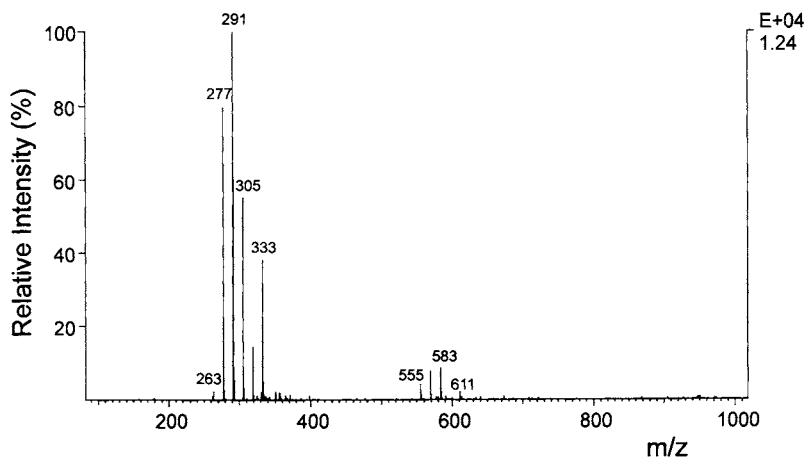
Fig. 19.21. ESI-FIA-MS(−) overview spectrum of secondary alkylsulfonate blend (SAS) (CH₃–(CH₂)ₙ–CH(SO₃H)–(CH₂)ₓ–CH₃) presenting [M − H]⁻ and [2M − H]⁻ ions [136].

without any other negatively ionizable compounds, with the exception of LAS [40, 81,88].

19.3.2.2 Alkane- and alkenesulfonates

Alkanesulfonates with the general formula C_nH_{2n+1}–SO_3H are detected nearly exclusively in the influents of biological sewage treatment plants [92,125,129]. Their appearance in effluents is an obvious sign of an operating trouble [129]. Secondary alkane sulfonates (SAS) with the modified formula CH_3–$(CH_2)_n$–$CH(SO_3H)$–$(CH_2)_x$–CH_3 ($n + x = 10$–15) are contained in surfactant formulations applied in household cleaning agents. This mixture of compounds was detected by FIA-MS in the negative APCI mode as [M − H]⁻ ions with m/z ratios at 263, 277, 291, 305, 319 and 333 [75]. The same peak shape can be recognized under ESI(−) conditions in solutions containing SAS in low concentrations. High concentrations of SAS, however, resulted in [M − H]⁻ ions at 263, 277, 291, 305, 319 and 333 and [2M–H]⁻ ions at 555, 569, 583, 597 and 611 [136] using APCI or ESI(−) ionization (Fig. 19.21).

The industrial blend of this mixture used for FIA-MS was separated by RP-C_{18} in combination with UV-DAD and MS detection. Since these compounds have no chromophor, UV detection must fail. The RIC together with the mass traces m/z 277, 291, 305 and 319 for ($n + x = 11$–14) recorded in the APCI(−) mode is shown in Fig. 19.22. The LC separation in the mass traces proves the large number of isomers of every SAS homologue because of the complex mixtures of precursors used for synthesis [136].

LC/ion spray mass spectrometry was applied for the separation of a synthetic mixture of alkylsulfonates (C_nH_{2n+1}–SO_3H; $n = 8$) and alkylsulfates with different alkyl chain lengths in the SIM ESI(−) mode [137]. Ion current profiles prove the separation of the compounds. The ionic constituents of the eluent were removed by a suppressor to improve the signal to noise ratio.

Alkenesulfonates as unsaturated compounds with the general formula C_nH_{2n-1}–SO_3H
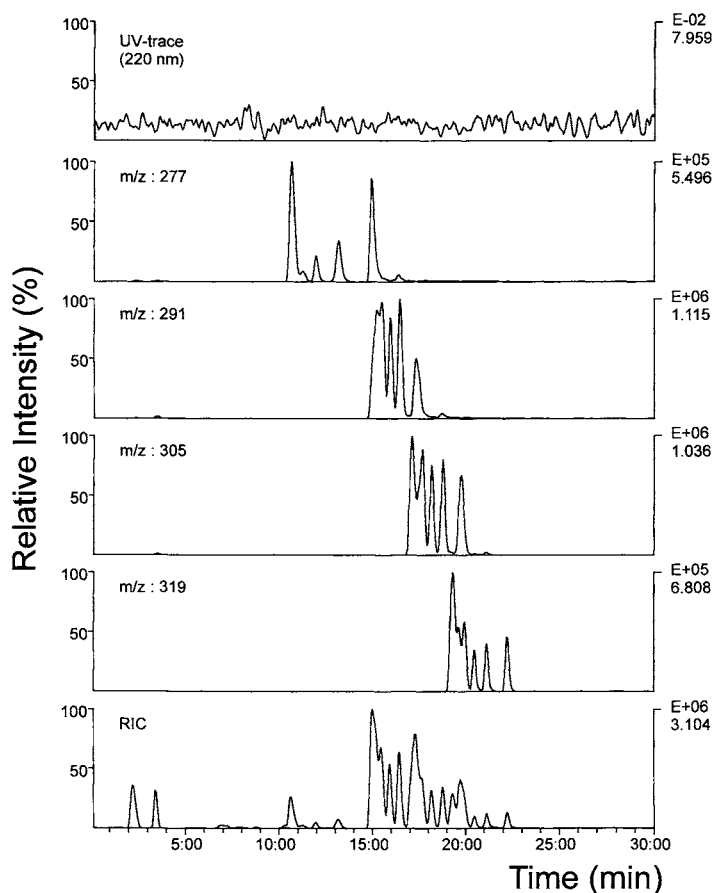
Fig. 19.22. APCI-LC-MS(−) total ion current chromatogram (RIC) and selected mass traces of SAS blend as in Fig. 19.21 and UV trace 220 nm [136].

were separated by RP-C_{18} column chromatography. APCI(−) was applied for ionization resulting in the total ion current and mass traces in Fig. 19.23. The commercial blend contained two homologues with C_{12} and C_{14} alkyl chains. These alkyl chains vary in structure, resulting in a complex mixture of signals in their mass traces. The compounds were ionized in the negative mode resulting in $[M − 1]^-$ ions at m/z 275 and 303. No UV-absorbance was found for these alkenesulfonates [136].

19.3.2.3 Alkylsulfates

Octadecyl sulfate ($C_{18}H_{37}$–O–SO_3^- Na^+) was examined by laser desorption fourier transform mass spectrometry (FT-MS) in the negative mode resulting in $[M − 1]^-$ ions. Little fragmentation was observed under these conditions [102].

LC/ion spray mass spectrometry was applied for the separation of a synthetic mixture of alkylsulfates (C_nH_{2n+1}–O–SO_3H; $n = 8, 10, 12, 14$ and 18) containing also alkylsulfonates
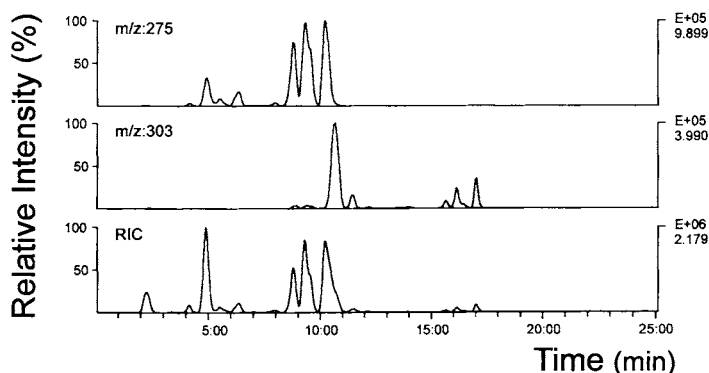
Fig. 19.23. APCI-LC-MS($-$) total ion current chromatogram (RIC) and selected mass traces (C_{12}-, C_{14}-homologues) of alkenesulfonates blend (C_nH_{2n-1}–SO_3H) [136].

(C_nH_{2n+1}–SO_3H) in the SIM ESI($-$) mode [137]. The separation of the compounds was checked by the ion current profiles. To improve the signal to noise ratio the ionic consti-tuents of the eluent were removed by a suppressor prior to MS detection. MS-MS data of alkylsulfates (see Section 19.4.2.4) were presented.

A blend of alkylsulfates (AS) with the general formula C_nH_{2n+1}–O–SO_3H was separated by RP-C_{18} column chromatography [134] combined with APCI($-$) ionization. The surfac-tant mixture consisted of the C_{12} and C_{14} homologues resulting in [M $-$ 1]$^-$ ions at m/z 265 and 293. Besides these ions adduct ions at m/z 363 and 391 can be observed in the mass
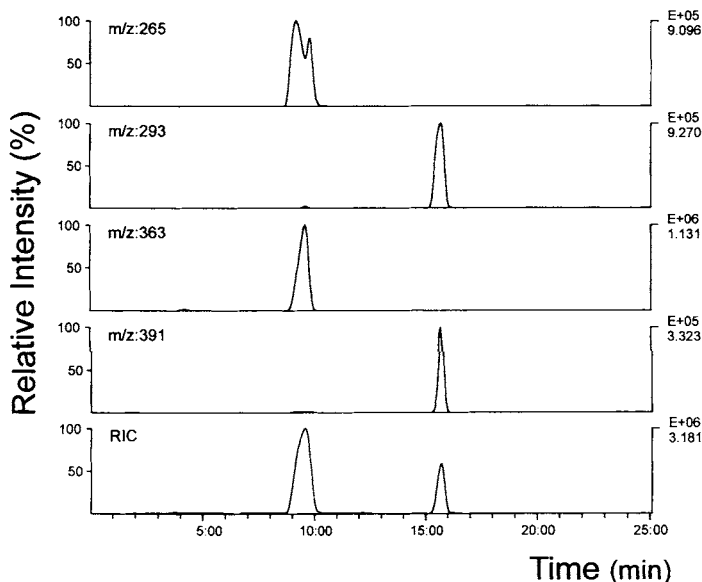
Fig. 19.24. APCI-LC-MS($-$) total ion current chromatogram (RIC) and selected mass traces of [M $-$ H]$^-$ ions and [M–H + 98]$^-$ adduct ions (C_{12}-, C_{14}-homologues) of alkylsulfate blend (C_nH_{2n+1}–O–SO_3H) [136].

traces in Fig. 19.24 [136]. These ions with $[M - 1 + 98]^-$ seem to be sulfuric acid adduct ions of the alkylsulfates ionized as $[M - 1]^-$ ions, too.

A method for monitoring individual species of the alkylsulfates (C_nH_{2n+1}–O–SO_3H) and alkylethersulfates (cf. Section 19.3.2.4) by ESI-LC-MS in the in- and effluent of a WWTP (Clermont County Lower East Fork, Milford, OH) and in the river water (Little Miami River) upstream of the WWTP was developed by Popenoe et al. [134] (see Section 19.5.1.2). RP-C_2 material was applied for concentration of the pollutants.

19.3.2.4 Alkylethersulfates

Laureth ether sulfate ($C_{12}H_{25}$–(O–CH_2–CH_2)$_n$–O–$SO_3^-H^+$) besides alkylsulfates, alkylsulfonates and dihexyl sulfosuccinate was examined by laser desorption fourier transform mass spectrometry (FT-MS) in the negative mode. All anionic surfactants with the exception of laureth ether sulfate gave one-peak mass spectra originating from the $[M - 1]^-$ ions of the sulfate or sulfonated compounds. Laureth ether sulfate gave a series of equally spaced ions ($\Delta m/z$ 44) because of a varying number of polyether units (PEG) in the molecules of the homologues ionized. Little fragmentation was observed under these conditions [102].

Alkylethersulfates (AES) (C_nH_{2n+1}–O–(CH_2–CH_2–O)$_x$–SO_3H) and the precursors from alkylethersulfate synthesis, the alkylsulfates as unreacted compounds were determined qualitatively using ESI-LC-MS(−). Detection of this compound mixture may cause problems. In spite of the very soft ionization procedure applying TSP ionization it was only possible to detect AES in the negative mode with reduced sensitivity. Positive ionization leads to the cleavage of the SO_3 moiety, pretending non-ionic surfactants of the polyether type (cf. Chapter 1) [59].

To elucidate this problem, extensive research was done [70]. For this purpose a commercial blend of AES was used for LC separation according to [134] to investigate the sensitivity and selectivity of the different API methods APCI and ESI in the positive and negative ionization mode. From water samples the AS as well as AES can be concentrated by RP-C_{18} SPE. Applying selective elution they are found in the methanol fraction [125]. The results confirm a high variability in the ionization efficiency for the different methods and modes applied.

As FIA-MS in the ESI(−) mode proved, the AES blend contained AES with alkyl chains with $n = 12$ and 14. Additionally the AS precursor compounds with $n = 12$ and 14 and $x = 0$ were present. In the AS and AES molecules the C_{12} and C_{14} alkyl chains were coupled with polyether moieties covering the number of $x = 0$–9 resulting in ions at m/z_{C12}: 265–661 and m/z_{C14}: 293–689, respectively.

In the APCI-FIA-MS(−) mode the same blend with its variety of homologues observed under ESI(−) conditions exhibits a single signal spectrum with the $[M - H]^-$ ion at m/z 337 generated from $C_{14}H_{29}$–O–CH_2–CH_2–O–SO_3H.

FIA-MS in the ESI(+) mode using ammonium acetate for ionization support of the AES blend resulted in a complex mixture of ions which were not yet completely identified. Under these conditions the intact molecules of AES were ionized as $[M + NH_4]^+$ ions.

In the positive APCI mode using ammonium acetate for ionization support, too, the AES were ionized as $[M + H-SO_3]^+$ ions [70].

To compare and elucidate the different ionization methods and detection modes, the
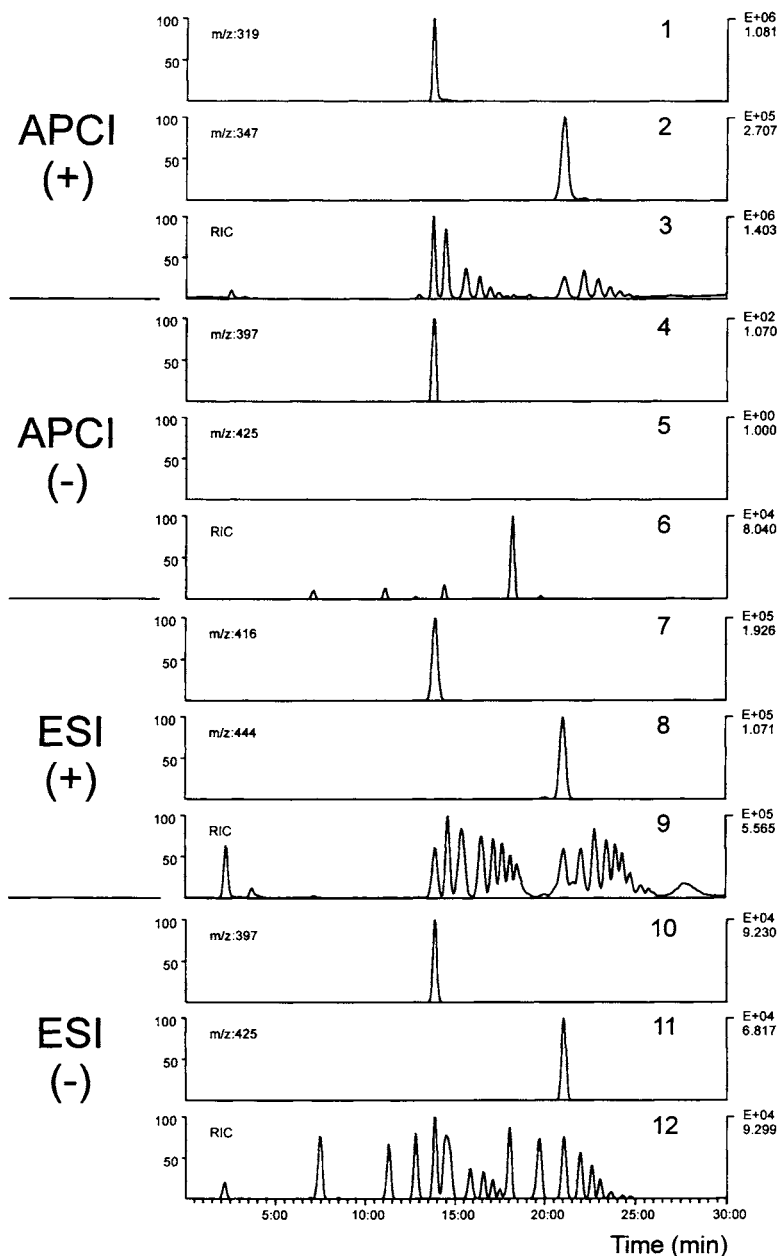
Fig. 19.25. APCI- and ESI-LC-MS total ion current chromatograms (RIC) and selected mass traces (C_{12}-, C_{14}-homologues) of alkylpolyethersulfate blend (C_nH_{2n+1}–(O–CH_2–CH_2)$_x$-OSO$_3$H) ionized in the positive and negative mode [70].

RICs of all LC separations and in parallel the mass traces of the C_{12} and C_{14} homologues containing a polyether chain of 3 units were combined in Fig. 19.25 [70]. MS recording

took place under standardized conditions. The results again demonstrate the quite large variation in the ionization efficiency of the interfaces applied for the ionization of the same compound mixture. As the comparison demonstrates, ESI($-$) was very effective in ionizing AES with the whole range of polyether units present in the mixture ($x = 1$–9) together with the AS ($x = 0$) (Fig. 19.25 12). APCI($+$) TICs (Fig. 19.25 3) started to exhibit signals in the range of $x \geqq 3$. The ions belonging to these signals, however, pretend ions of alkylpolyglycolethers because of a loss of the SO_3 moiety. In contrast to this positive ionization using ESI (Fig. 19.25 9) resulted in $[M + NH_4]^+$ ions. Negative APCI (Fig. 19.25 6) is not applicable for determination of AES because of a tremendous loss of sensitivity [70].

Anionic surfactants of the AES type were detected qualitatively in the ESI-FIA-MS mode in the mixture of a formulation with the label 'ecologic'. The presentation of the TIC after HPLC separation in the form of a 'contour plot' allowed the identification of the AES in parallel with an amphoteric surfactant of alkylamidopropylbetaine type [96] (see Section 19.3.4.2).

AES were determined besides alkylsulfates qualitatively and quantitatively (see Section 19.5.1.2) by ESI-LC-MS in the in- and effluent of a WWTP (Clermont County Lower East Fork, Milford, OH) and in the river water (Little Miami River) upstream of the WWTP. A method for monitoring individual species of these compounds in environmental samples was developed by Popenoe et al. [134]. For concentration of the pollutants from water samples SPE using RP-C_2 material was applied. For elution of the SPE material a mixture of methanol/iso-propanol (80/20, v/v) was used. LC with ESI-MS($-$) was performed on a RP-C_8 column. As standard materials for confirmation and calibration the deuterated sodium salt of the mono EO-AES ($C_{12}D_{25}$–O–CH_2–CH_2–O–SO_3Na) and a commercial blend of $C_{12-15}EO_{12}S$ AES in the negative mode and PPG in the positive mode were applied [134].

Research samples of propoxy/ethoxy sulfate containing a mixture of various alkyl chain lengths and ethoxylate (EO) and propoxylate (PO) groups with the general structure (C_nH_{2n+1}–(O–$CH(CH_3)$–CH_2)$_x$–(O–CH_2–CH_2)$_y$–O–$SO_3^-Na^+$) were investigated by ESI-FIA-MS($+/-$) and APCI-FIA-MS($+$) [132]. Qualitative and quantitative information was desired. The mass spec was operated with a variation of the source and probe temperature. ESI($+$) produced $[M + Na]^+$ ions from alkylsulfate whereas the EO/PO alkylsulfates were ionized as $[M + Na]^+$ ions and as desulfated $[M + NaSO_3 + H + Na]^+$ ions, which made the interpretation of the spectra more difficult. In contrary ESI($-$) ionization resulted in one series of $[M - Na]^-$ ions enabling qualitative and semi-quantitative analysis (see Section 19.5.1.2). APCI($+$) produced no molecular ions for alkylsulfate, however, predominantly the $[M-NaSO_3 + 2H]^+$ ions for the EO/PO alkylsulfates were generated. These compounds produce complex FIA-MS spectra because of overlapping of the surfactant homologues synthesized by a more or less untargeted condensation of the precursor compounds. To overcome this problem, LC separation prior to FIA-MS is necessary [132].

19.3.2.5 Alkylethercarboxylates

The alkylethercarboxylates with the general structure (C_nH_{2n+1}–O–(CH_2–CH_2–O)$_x$–CH_2–COO^-H^+) today are used more frequently in households for cleaning purposes.
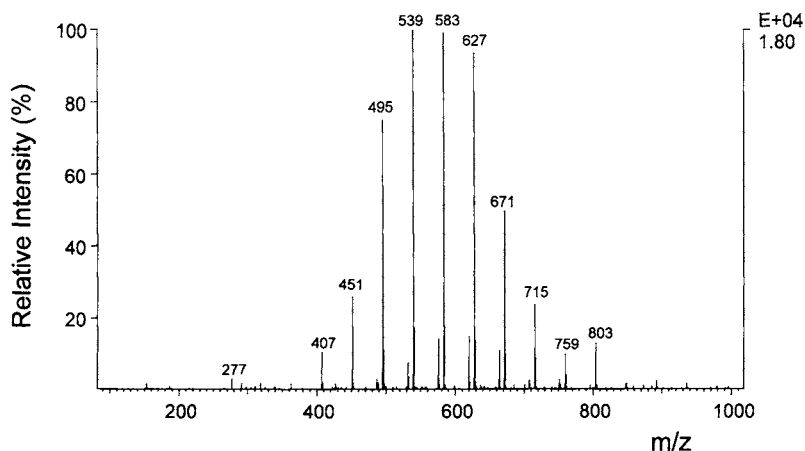
Fig. 19.26. ESI-FIA-MS(−) overview spectrum of alkylpolyethercarboxylate blend (C_8H_{17}–O–(CH_2–CH_2–O)$_n$–CH_2–COO^-; n = 5–15) ionized as [M − H]$^-$ ions [136].

Since this type of anionic surfactant seems to be easily eliminable in the waste water treatment process, it was never found in effluents of WWTPs. Moreover, it is very difficult to detect this surfactant type even in the influent of WWTPs because of the complex matrix of untreated sewage. This surfactant mixture can be concentrated from waste water samples applying C_{18} SPE and selective elution. The compounds can be detected in the methanol/water and methanol fractions by MS [125]. The structure shown in Fig. 19.18 II demands mass spectra like non-ionic surfactants of the polyether type with equally spaced signals ($\Delta m/z$ 44). Ionization of a commercial blend of this surfactant mixture in the presence of acetic acid is possible in the negative as well as positive mode by APCI or ESI. The APCI and ESI FIA-MS(−) spectra contain the same deprotonated molecular ions ([M − H]$^-$) as presented for the ESI spectrum in Fig. 19.26, representing the equally spaced ions of the C_8H_{17}–O–(CH_2–CH_2–O)$_n$–CH_2–COO^- homologues (n = 5–15; m/z 407–803) [136]. The same mixture ionized in the positive ESI mode contains a complex mixture of adduct ions ([M + Na]$^+$, [M + NH$_4$]$^+$) and ions of alkylpolyglycolethers, as MS-MS proves. Either these carboxylates show a loss of CO_2 under ESI(+) ionization, or alkylpolyglycolether as byproducts or precursors from synthesis are found in the positive mode. However, APCI(+) shows a clearer FIA spectrum than ESI(+), but in parallel with a loss of sensitivity [136].

The RP-C_{18} LC separation in combination with APCI(+) ionization for the alkylethercarboxylates was performed and is shown in Fig. 19.27. The inset with the MS spectrum of peak 2 demonstrates the separation efficiency from by-products and precursor compounds (PEG (peak 1) and C_nH_{2n+1}–O–(CH_2–CH_2–O)$_x$–H (peak 3, n = 8; peak 4, n = 10) [136].

19.3.2.6. Alkylarylethersulfates, -sulfonates, -phosphates and di-alkylarylethercarboxylates

Besides alkylphenolethoxylates as nonionic surfactants the sulfates (alkylphenolether-
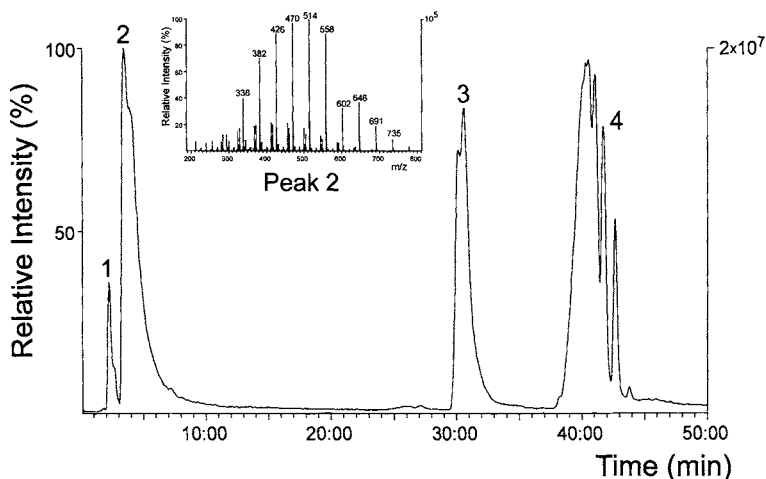
Fig. 19.27. APCI-LC-MS(+) total ion current chromatogram of alkylpolyethercarboxylate blend as in Fig. 19.26 confirming the separation of the alkylpolyethercarboxylates from impurities by its mass spectrum in the inset [136].

sulfates, $C_nH_{2n+1}-C_6H_4-O-(CH_2-CH_2-O)_m-SO_3^-$), sulfonates (alkylphenolethersulfonates, $C_nH_{2n+1}-C_6H_4-O-(CH_2-CH_2-O)_n-CH_2-SO_3^-$), phosphates (alkylphenoletherphosphates, $C_nH_{2n+1}-C_6H_4-O-(CH_2-CH_2-O)_m-PO(OH)O^-$) and carboxylates (di-alkylphenolethercarboxylates ($(C_nH_{2n+1})_2-C_6H_3-O-(CH_2-CH_2-O)_m-CH_2-CO_2^-$) were produced and applied. These compounds are anionic surfactants with the structural element 'alkylphenolether' which is coupled with $-O-SO_3^-$, $-SO_3^-$, $-O-PO(OH)O^-$, $-COO^-$ moieties. The alkyl chains of these compounds normally contained eight or nine carbon atoms. An exception was found for the compound mixture of carboxylates, consisting of di-nonylphenol moieties. This di-alkylphenolethercarboxylate compound exists as industrial blend [136], whereas the monoalkylphenol-ethercarboxylates are known as biodegradation products of the APEOs [32,105,138].

The di-nonylphenolethercarboxylates ($(C_9H_{19})_2-C_6H_3-O-(CH_2-CH_2-O)_m-CH_2-CO_2^-$) were examined as industrial blend together with the sulfate, sulfonate and phosphate compounds. The sulfates, phosphates and carboxylates of this type of anionic phenolethers in parallel to the aliphatic ethers show equally spaced signals with $\Delta m/z$ 44 in the FIA-MS spectra. But according to the ionization method applied – APCI or ESI in the positive and negative mode – equally spaced signals came either from the anionic compounds themselves or from the alkylphenolether ions after bond cleavage between polyether chain and acid groups ($-O-SO_3^-$, $-O-PO(OH)O^-$, $-CH_2-CO_2^-$) during ionization. In addition to the alkylphenol polyether ions complex mixtures of adduct ions could be observed in the positive mode, whereas all compound could be ionized by negative ESI-FIA-MS without any decomposition. Under this condition the compounds exhibit $[M - H]^-$ ions, and it became obvious that the sulfonate compound (Triton® X 200) was a defined molecule type with the formula $C_9H_{19}-C_6H_4-O-(CH_2-CH_2-O)_2-CH_2-SO_3^-$). Under ESI-FIA-MS(−) conditions this $[M - H]^-$ ion at m/z 401 could be observed, and ESI in the positive
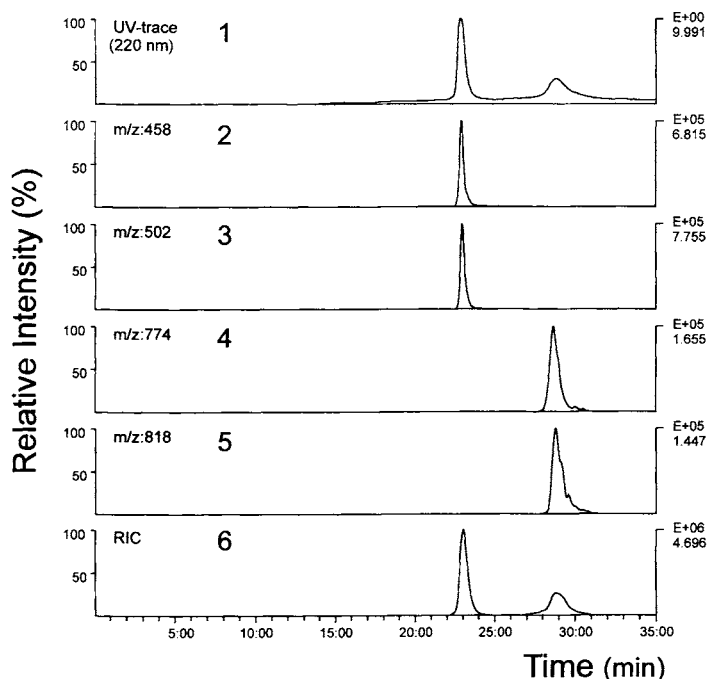
Fig. 19.28. APCI-LC-MS(+) total ion current chromatograms (RIC; 6) and selected mass traces of nonylphenolethoxylates (NPEO; 2,3) and di-nonylarylpolyethercarboxylates (4,5) and UV trace 220 nm (1) [136].

mode in the presence of ammonium acetate resulted in $[M + NH_4]^+$ and $[M–SO_2 + NH_4]^+$ ions at 420 or 356, respectively [136].

In the APCI-LC-MS(+) TIC and mass traces separating nonylphenolethoxylate (NPEO) and di-nonyl-phenolpolyethoxycarboxylate (DNPEC) on a RP-C_{18} column in the presence of ammonium ions, both compounds could be detected with different retention behavior (see Fig. 19.28). Under these conditions the NPEO homologues exhibit $[M + NH_4]^+$ ions, whereas the DNPEC were ionized as $[M–(CH_2–CO_2) + NH_4]^+$ pretending a non-ionic surfactant mixture of the polyether type [136].

19.3.2.7 Fluorinated phoshinic and phosphonic acid derivatives

A mixture of derivatives of phosphonic acid $C_nF_{2n+1}–P(O)(OH)_2$ and phosphinic acid $C_nF_{2n+1}(C_mF_{2m+1})–P(O)OH$ ($n = l \neq m$) containing perfluoro alkyl chains [59,139], used as anionic surfactants, was examined by negative ESI- and APCI-FIA-MS. By analogy with their behavior in the TSP-FIA-MS(−) process, the phosphonic acid forms $[M − H]^-$ ions at m/z 399, 499 and 599 representing a perfluoroalkyl chain with $n = 6$, 8 or 10 carbon atoms [136]. The signals appearing at m/z 799 and 899 under TSP(−) conditions [59], however, could not be found under ESI and APCI ionization, but signals at m/z 701, 801 and 901 generated by the phosphinic acid now could be observed. The equally spaced signals with $\Delta m/z$ 100 could be explained by the different chain lengths of the fluorinated
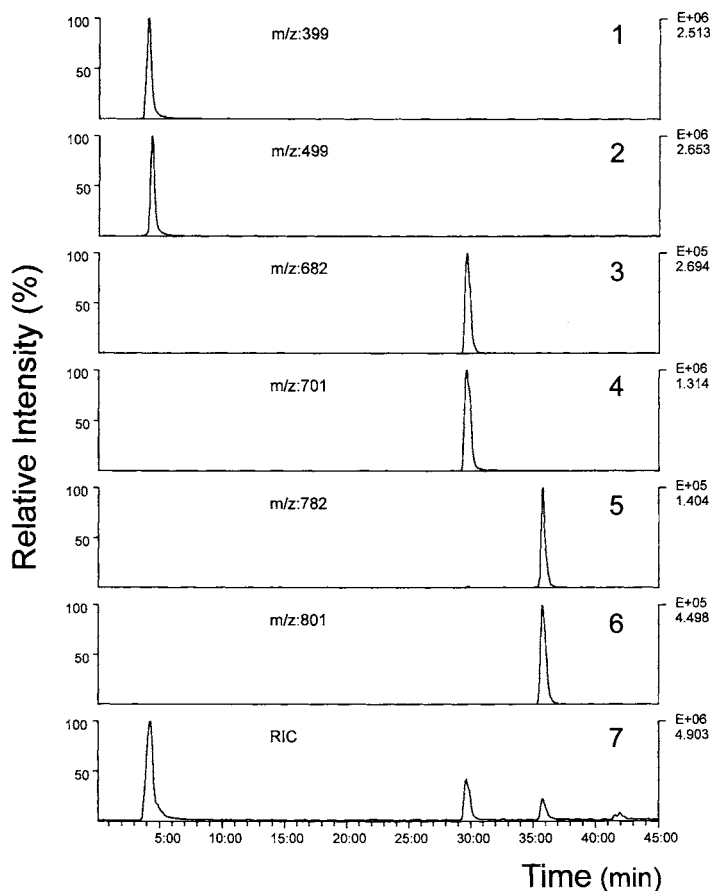
Fig. 19.29. APCI-LC-MS($-$) total ion current chromatograms (RIC; 7) and selected mass traces of anionic fluorinated phoshinic and phosphonic acid surfactant mixture (C_nF_{2n+1}-P(O)(OH)$_2$ and $C_nF_{2n+1}(C_mF_{2m+1})$-P(O)OH) [136].

alkyl groups representing always 100 u for every (CF$_2$)$_2$ unit. In addition to these molecular [M $-$ H]$^-$ ions of the phosphinic acid the APCI-FIA-MS($-$) provides ions with a *m/z* ratio of 682, 782 and 882. These ions, which appear in LC separation together with the ions at *m/z* 701, 801 and 901 and differ to the phosphinic acid with $\Delta m/z$ 19, seem to be reaction products of the ionization process. They will be generated after LC separation in the APCI($-$) process [136]. Positive ionization was impossible in both techniques, ESI as well as APCI.

LC-MS in the APCI($-$) mode resulted in an excellent separation of the two component mixtures and partly of their homologues, as demonstrated by mass trace analysis (see Fig. 19.29) [136].

19.3.2.8 Sulfosuccinates

Sulfosuccinates are applied as surfactants for personal hygiene because of their hypoal-
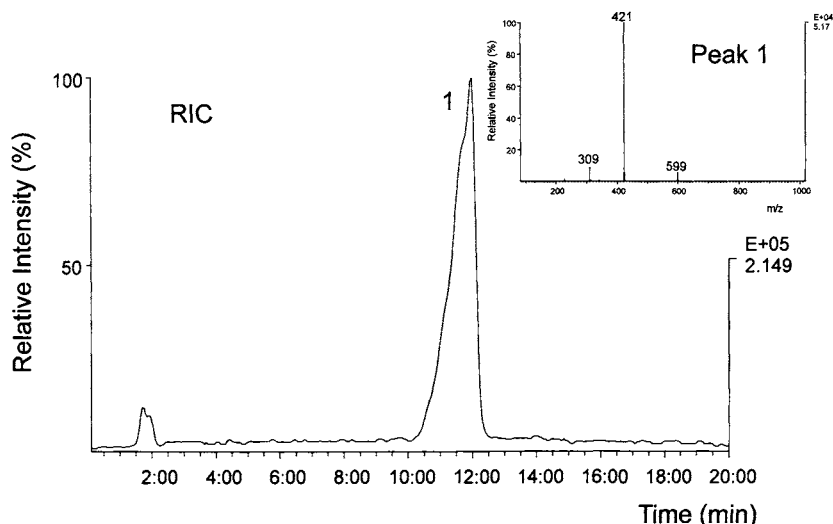
Fig. 19.30. ESI-LC-MS(−) total ion current chromatogram of sulfosuccinate sodium salt $C_8H_{17}OOC-CH-(SO_3^-)-CH_2-COOC_8H_{17}$ Na^+ confirming the purity of the compound in the inset [136].

lergenic features. The sodium salt of the sulfosuccinate with the formula $ROOC-CH-(SO_3^-)-CH_2-COOR$ Na^+ ($R = C_8H_{17}$) was examined by APCI-FIA-MS in the positive and negative mode. The addition of ammonium acetate resulted in $[M - NH_4]^+$ ions with m/z 440 applying APCI(+) and $[M - H]^-$ ions with m/z 421 in the APCI(−) mode. The mixture was very pure consisting of only one compound, as the ESI-LC-MS(−) TIC in combination with the mass spectrum under the signal in Fig. 19.30 proves [136].

Dihexyl sulfosuccinate ($C_6H_{11}OOC-CH-(SO_3^-)-CH_2-COOC_6H_{11}$ Na^+) was examined by laser desorption fourier transform mass spectrometry (FT-MS) in the negative mode resulting in $[M - 1]^-$ ions. No fragmentation was observed under these conditions [102].

19.3.2.9 Alkylpolyglucoside esters

An API-MS method for the quality control of the production process for anionic surfactants of the alkylpolyglucoside ester type is presented. Alkylpolyglucoside esters from sulfosuccinic, citric and tartric acid were determined by FIA-ESI-MS(+/−). In the positive ionization mode sodium or ammonia adduct ions ($[M + Na]^+$, $[M + 2Na]^+$ or $[M + NH_4]^+$) of the esters will be produced from the anionic surfactants. The results were complex spectra. This could be avoided by negative ionization producing predominantly deprotonated molecular ions ($[M - H]^-$). The spectra obtained in the negative mode were simpler providing direct characterization of the compounds without prior LC separation [140].

19.3.3 Cationics

The ionization of cationic surfactants in the APCI(−) or ESI(+) mode is supported by
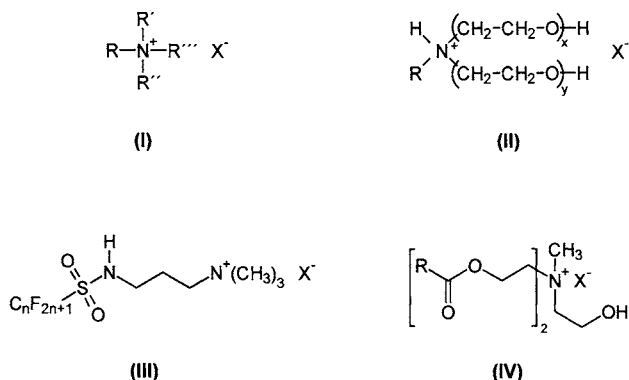
Fig. 19.31. I–IV. Structures of selected cationic surfactants: (I) quaternary ammonium compounds; (II) alkyl polyglycol amines; (III) quaternary perfluoralkyl ammonium compounds; (IV) dialkylcarbooxyethyl hydroxyethyl methyl ammonium compounds (esterquats).

the nitrogen atom contained in most of the cationics. However, the detection in water samples is complicated because of their low concentration in the water phase. There are different reasons for this fact: firstly, cationics belong to those surface active compounds which are not applied in such large quantities compared to non-ionic or anionic surfactants and therefore cannot be found in such high concentrations (consumption of cationics: about 250 000 tons in 1986 [34]). Secondly, cationics always try to bind to surfaces, i.e. their elimination from the water phase takes place by adsorption to soils, sediments and suspended particles in the water because of hydrophobic and electrostatic interactions with surfaces that are negatively charged. Their liquid/liquid extraction takes much time and sometimes is incomplete because of their adsorptive tendency, even when organic solvents with high polarity and extraction potential are used. However, this problem with the extraction of cationics from these matrices can be solved, applying supercritical fluid extraction [34]. In parallel this technique saves time. Another effective method is SPE, but if water samples are filtered prior to SPE, the cationics should also be extracted from the filter materials. The elution of cationics from SPE materials is effective with all organic eluents. The fraction received by freeze-drying after SPE never contained any of these compounds [125].

Compounds like primary, secondary, tertiary and quarternary ammonium salts are applied as typical cationic surfactants besides benzylalkyl ammonium salts and ethoxylated quaternary amines or ester quats. Furthermore pyridine-, imidazoline-, oxazoline-, thiazoline-, sulfonium- and tropylium salts belong to this type of surfactants. A number of selected cationics with high production rates is presented in Fig. 19.31. In many cases their bacteriostatic potential is an important feature and essential for their application. However, their toxicity for aquatic organisms [2,3] in parallel makes them ecotoxicologically more relevant compared to other surfactant types.

Although cationic surfactants show a good response in the API ionization process, up to now there are only few data in literature concerning the application of APCI- and ESI-FIA-MS or -LC-MS for their determination [34,76].

19.3.3.1 Quaternary alkyl ammonium compounds

So-called quats (quaternary ammonium compounds) with varying alkyl chains are not only used because of their surface activity but also as powerful counter ions in ion-pairing chromatography. Factors affecting the ionization efficiency of quaternary ammonium compounds were examined, using FIA-MS in the positive ESI mode [141]. The results obtained demonstrated that quaternary ammonium compounds containing only alkyl groups give the best sensitivity under these conditions. In contrast to FAB (fast-atom bombardment) and TSP giving rise to dealkylation reactions, whereas ion spray spectra show one single peak as $[M]^+$ ions. Basic research results about the efficiency in ionization were reported [141].

Ion chromatography in combination with ion spray mass spectrometry was (ESI–IC–MS) applied for the separation of a synthetic mixture of industrially important quaternary ammonium compounds in the ESI($+$) mode [137]. The mixture contained four symmetrical tetraalkylammonium compounds varying in the alkyl groups starting at C_3H_7 (propyl) and ending at C_6H_{13} (hexyl). Ion current profiles reveal the chromatographic integrity and signal response by the mass specrometer used. The ionic constituents essential for the elution in the separation process were removed by a suppressor to improve the signal to noise ratio prior to MS detection. Fig. 19.32 demonstrates the results [137]. MS-MS data of quaternary ammonium compounds (see Section 19.4.3) were presented.

The homologue quarternary ammonium compounds of the general formula $RR'N^{\oplus}(CH_3)_2X^-$ with R = C_nH_{2n+1} ($n = 12$, 14 and 16), R$'$ = benzyl (C_6H_5–CH_2–) and X$^-$ = acetate was examined by ESI-FIA-MS($+$) resulting in $[M]^+$ ions at m/z 304, 332 and 360. This commercial blend was also analyzed by APCI-FIA-MS($+$), giving rise to dealkylations or Hoffmann-type elimination reactions as reported for other quats in literature [141]. Ions detected under these conditions proved either an elimination of CH_2 or the cleavage of the benzyl moiety resulting in ions at m/z 290, 318 and 346 ([M $-$ $CH_2]^+$) or 214, 242 and 256 ($[M]^+ - C_6H_5$–CH_2–), respectively [67].

The surfactant mixture, declared as alkyldimethylbenzylammoniumacetate, investigated by APCI-FIA-MS($+$) showed the same behavior as obtained under TSP($+$) conditions [59].

Blends of quaternary ammonium surfactants (quats) with the general formula $(R)_nN^{\oplus}(CH_3)_{4-n}$, where R can be alkyl chains of 12 to 22 or more carbon atoms, were studied by ESI-FIA-MS($+$). These compound applied in products for personal hygiene were analyzed according to their chain length distribution. Quantification (see Section 19.5.1.3) in commercial products and amounts of quats deposited on hair was carried out. Identification by MS-MS was done (see Section 19.4.3) [76].

The quarternary ammonium cationic surfactant ditallowdimethylammonium (DTDMAC), used as softener in high amounts, was determined after SFE of digested sludge in the extracts using normal phase HPLC with fluorescence detection after post column derivatization and by ESI-FIA-MS($+$). The results were checked by commercially available DTDMAC. The DTDMAC mixture shows ions at m/z 495, 523 and 551 for the compounds $RR'N^{\oplus}(CH_3)_2 X^-$ (R = $l \neq$ R$'$) as shown in Fig. 19.33 [34].

The examination of a fluorine-containing cationic surfactant of quat type with the general formula C_nF_{2n+1}–SO_2–NH–CH_2–CH_2–CH_2–$N^{\oplus}(CH_3)_3X^-$ by FIA-MS using APCI and ESI in the positive and negative mode resulted in a dealkylation with ions at
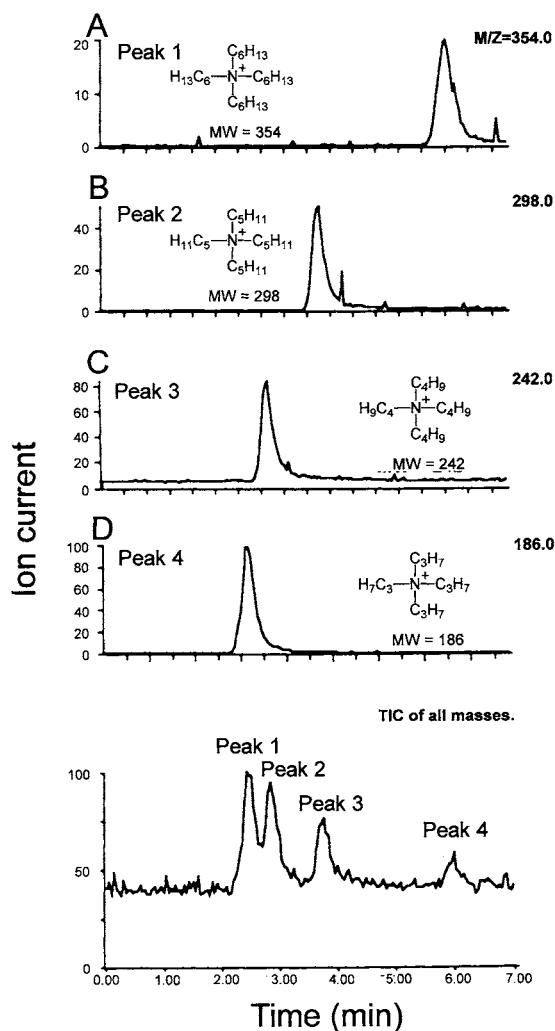
Fig. 19.32. ESI-IC-MS(+) total ion current trace (TIC) and selected mass traces of an industrial blend of quaternary ammonium compounds. Reproduced with permission from [137]. © 1990 by American Chemical Society.

m/z 585 or 583, respectively, if APCI was used and the alkyl chain contained the moiety C_8F_{17} [67]. The ions generated under these conditions were dealkylation products of [M − $CH_2]^+$ or [M − H–$CH_3]^-$ type as reported in literature [141]. ESI(+), however, mainly produced the [M]$^+$ ions at *m/z* 599 besides a small part of dealkylated ions [M − $CH_2]^+$ at 585, whereas ESI(−) produced the identical ions as found under APCI(−) conditions. This compound was the first cationic surfactant that could be ionized in negative mode, although it contained ammonium nitrogen [67].
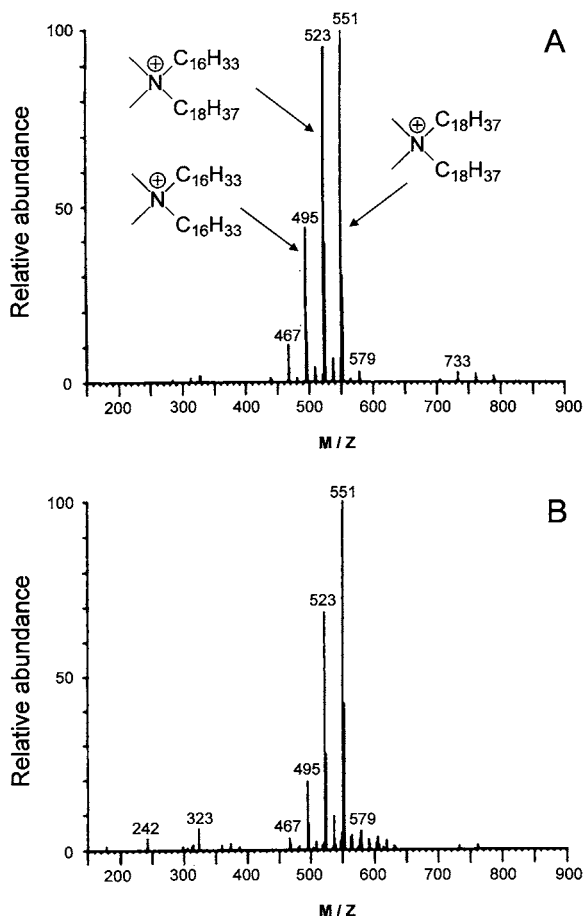
Fig. 19.33. ESI-FIA-MS(+) overview spectra of (A) SFE extract of waste water sludge and (B) of an industrial blend of quaternary ammonium compounds. Reproduced with permission from [34]. © 1996 by American Chemical Society.

19.3.3.2 Quaternary carboxyalkyl ammonium compounds

Industrial blends of quaternary carboxyalkyl ammonium compounds (so-called 'ester-quats') with the general formula $(R(CO)OCH_2CH_2)_2–N^{\oplus}(CH_3)CH_2CH_2OH$ X^- (R = mixture of alkyl (C_{13}, C_{15} and C_{17} = tallowyl) or unsaturated alkenyl ($C_{17}H_{33}$ = oleyl) moieties) were examined using APCI- and ESI-FIA-MS. These compounds today were applied as softeners for the substitution of the quaternary alkyl compounds especially DTDMAC known as hardly degradable [93] and toxic [2,3]. Ionization of the dioleyl compound in the positive mode was possible with both types of interfaces, however, APCI-FIA-MS(+) resulted in a very complex spectrum showing a large number of fragment ions, whereas ESI(+) generated $[M]^+$ ions of the compounds with R = $C_{17}H_{33}$ and of compounds containing a mixture of $C_{17}H_{33}$ and $C_{15}H_{29}$ moieties as impurity of the blend as
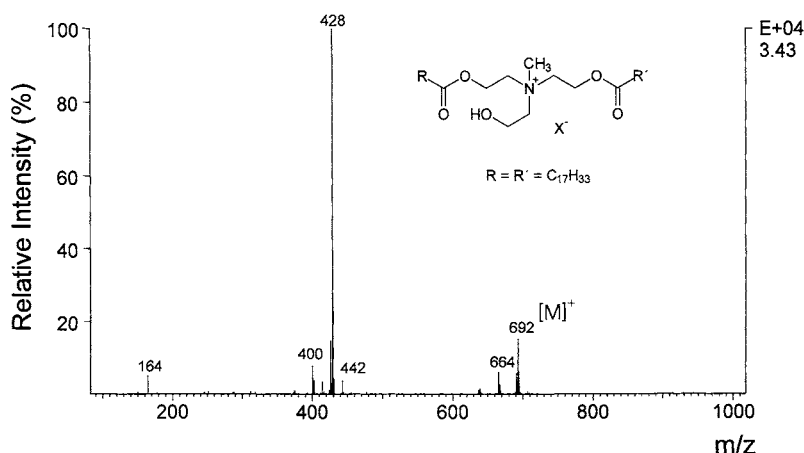
Fig. 19.34. ESI-FIA-MS(+) overview spectrum of industrial blend of an quaternary carboxyalkyl ammonium compound (esterquat) with the general formula $(R(CO)OCH_2CH_2)_2-N^{\oplus}(CH_3)CH_2CH_2OH\ X^-$ ($R = C_{17}H_{33}$ = oleyl) [142].

shown in Fig. 19.34. Besides these molecular ions at m/z 692 and 664 the fragment ions at m/z 400 and 428 as a result of the abstraction of $C_{17}H_{33}COO$ could be observed under standard ionization conditions [142].

19.3.3.3 Fatty acid polyglycol amines

The qualitative and quantitative surfactant contents of a WWTP discharge, surface water and foam resulting from an overflow drop were determined in a series of surface water examinations of a tributary of the Elbe river [81]. FIA- APCI(+/−) was applied using ammonium acetate for ionization support. Besides non-ionic surfactants of alkyl- and arylethoxylate type with different polyether chain lengths, the cationic surfactants of fatty acid polyglycol amine type with the general formula $(R-N^{\oplus}H((CH_2-CH_2-O)_{x,y}H)_2X^-)$ were determined qualitatively and were quantified (cf. Section 19.5.1.3). For concentration purposes SPE using RP-C_{18} in combination with selective elution was used [40,88]. After a sequential selective elution [18,59,92] with hexane/ether, ether, methanol/water and methanol the pollutants in the fractions were ionized. The pattern of equal-spaced signals ($\Delta m/z$ 44) starting at m/z 318 and ending at 758 (cf. Section 19.3.1) Fig. 19.3a–c provides an excellent overview concerning the more or less selective elution efficiency [40,88]. The polyglycol amines (★) appear as $[M + H]^+$ ions. A coelution effect, resulting from the high surface activity of the alkyl polyglycol amines, could be observed under methanol/water elution conditions. The polyglycol amines (★) with short polyglycolether chains could be observed in the FIA-APCI-MS(+) spectra of this methanol/water eluate. The alkyl polyglycol amines with long ether chains and elevated m/z ratio dominate the methanol fraction (Fig. 19.3c) [40,88].

LC separation in the RP-C_{18} mode was impossible because the alkyl polyglycol amines could not be eluted under these chromatographic separation conditions [88].

$$R-\overset{R'}{\underset{R''}{\overset{|}{N}}}{}^{+}-O^{-} \qquad R-\overset{R'}{\underset{R''}{\overset{|}{N}}}{}^{+}-(CH_2)_x-C\overset{O}{\underset{O^-}{\diagup}} \qquad R-\overset{R'}{\underset{R''}{\overset{|}{N}}}{}^{+}-(CH_2)_x-SO_3^{-}$$
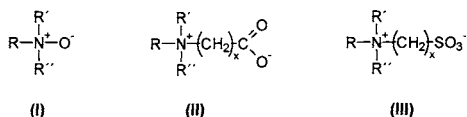
(I)   (II)   (III)

Fig. 19.35. I–III. Structures of selected amphoteric surfactants: (I) alkyl amine oxides; (II) betaines; (III) sulfo betaines.

19.3.4. Amphoterics

Surfactants with an amphoteric behavior normally are very well biodegradable and therefore their ecotoxicological relevance can be regarded as very low. Since these compounds are very well biodegradable, they should be contained in environmental samples only in special cases. This is also the reason why only few results from ESI- und APCI-MS examinations of amphoteric surfactants have been published up to now. The pursuit of amphoterics in their production process by MS, however, may help to optimize the purity of the synthetic products, so that detection of amphoterics mainly is applied in this field. In addition the investigation of formulations of surfactants from competitors is a very important task. A selection of amphoterics of relevance because of their production rates is presented in form of their structural formulas in Fig. 19.35.

19.3.4.1 Amine oxides

Amine oxides show amphoteric behavior. They are known to be very well biodegradable, and since they have hypoallergenic features, they are used for products for personal care like shampoos and shower gels. The oxidation of amines resulted in compounds with the formula $RR'R''N^{\oplus} \rightarrow O^-$. Normally two methyl groups are bonded to the nitrogen ($R' = R'' = CH_3$) whereas R varies because of its origin from petrochemicals. The ionization of the industrial blend Genaminox CS in the APCI and ESI-FIA-MS(+) mode generates ions of $[M + H]^+$ type with m/z 230 for $R = C_{12}H_{25}$. The homologues are equally spaced with $\Delta m/z$ 28. The maximum chain length was C_{18} at m/z 314. In parallel to these monomeric ions generated, dimeric ions can be observed under the conditions applied. A statistical partition of the possible combinations of amine oxides was found in the ions starting with the dimeric ion with m/z 460 which represents $[(C_{12}H_{25}(CH_3)_2N^{\oplus} \rightarrow O)_2]^-$ and ending at m/z 628 for the dimeric ion $[(C_{18}H_{27}(CH_3)_2N^{\oplus} \rightarrow O)_2]^-$ [142].

19.3.4.2 Betaines

The presentation of the TIC after HPLC separation in the form of a 'contour plot' allowed the identification of an amphoteric surfactant of alkylamidopropylbetaine type [96] in parallel with the anionic surfactant mixture of AES (see Section 19.3.2) in the ESI-FIA-MS(+) mode. The mixture separated by LC was a formulation with the label 'ecologic'.

The industrial blend of a betaine with the general formula $C_{11}H_{23}-C(O)-NH-(CH_2)_3-N^{\oplus}((CH_3)_2)-CH_2-COO^-H^+$ and a molar mass of 342 was examined by APCI- and ESI-

FIA-MS(+/−). This mixture of homologues show the same behavior as the sulfobetaine C_nH_{2n+1}–C(O)–NH–$(CH_2)_3$–$N^{\oplus}((CH_3)_2)$–CH_2–CH(OH)–CH_2–SO_3^- under ESI(+) conditions, i.e. $[M + H]^+$ ions with m/z 343 in the positive mode can be observed for the compound with $n = 11$. Under ESI(−) conditions, however, an unidentified adduct ion at m/z 377 ($n = 11$) and homologues with $\Delta28$ according to the length of the alkyl chain can be observed [142].

The ESI-LC-MS(+) separation was as successful as for the sulfobetaine mixture. Five homologue compounds from the mixture with m/z ratios of 287–399 ($\Delta m/z$ 28) were observed [142].

The detection of an industrial blend of the amphoteric surfactants 3-(3-cocoamido-propyl)-dimethylammonium-2-hydroxypropane-sulfonate (cocoamidopropyl hydroxysul-taine) has been studied using APCI- and ESI-FIA-MS in the positive and negative mode [142]. The sulfobetaine structure according to the systematic name cited before has the general formula $C_{11}H_{23}$–C(O)–NH–$(CH_2)_3$–$N^{\oplus}((CH_3)_2)$–CH_2–CH(OH)–CH_2–SO_3^- and a molar mass of 422. As a molecule containing a nitrogen atom $[M + H]^+$ ions with m/z 423 in the positive or $[M − H]^-$ ions with m/z 421 in the negative mode were expected. However, ionization results were quite different. APCI(−) ionization failed completely, whereas APCI(+) ionization generated $[M − (CH_2–CH(OH)–CH_2–SO_3)]^+$ ions at m/z 285 as dominating ions. Besides these compounds some more ions of this type of homologues with different alkyl chain length ($\pm\Delta28$; –CH_2–CH_2-) could be detected, i.e. bond cleavage took place for all homologues under APCI(+) conditions. In contrast to this behavior ESI(+/−) resulted in molecule adduct ions of $[M + H]^+$ type with m/z 423 for positive ionization, whereas ESI-FIA-MS(−) ionization generated $[M + acetate]^-$ ions at m/z 481.

The ESI-LC-MS(+) TIC and the mass traces of the ions with equally spaced molar masses ($\Delta28$ u) prove an excellent separation of the homologues with molecular ions of 367, 395, 423, 451 and 479 in Fig. 19.36 [142].

19.3.5 Metabolites

The biochemical degradation of anthropogenic surfactants is clearly distinguished in primary degradation and ultimate degradation. The primary degradation of surfactants should be connected with the loss of their surface activity, whereas ultimate degradation means mineralisation, i.e. transformation of the surfactant molecule into inorganic components such as H_2O, CO_2 and salts. However, metabolic processes may also generate compounds with surface activity. Some of these compounds or their homologues may be in parallel synthetic products used as anionic surfactants like the alkylphenolether carboxylates (cf. Section 19.3.2.6). But metabolites from non-surface-active compounds may also be surface-active after a transformation process during waste water treatment or other degradative processes in the aquatic environment. Their presence in the WWTP causes problems because of foam in the effluents combined with organic matter which has not been eliminated in the settling tanks. These problems were often described as problem of bacteria biocoenosis (*norcadia, spherotilus natans* etc.), but this may be only one aspect besides others such as the presence of anthropogenic and biogenic surfactants. First results in the detection and identification of biogenic surfactants are now available [80], however, substance-specific API-MS methods have to be applied. We know from the detection of surfactants by substance-class-specific methods like measurement of the methylene blue
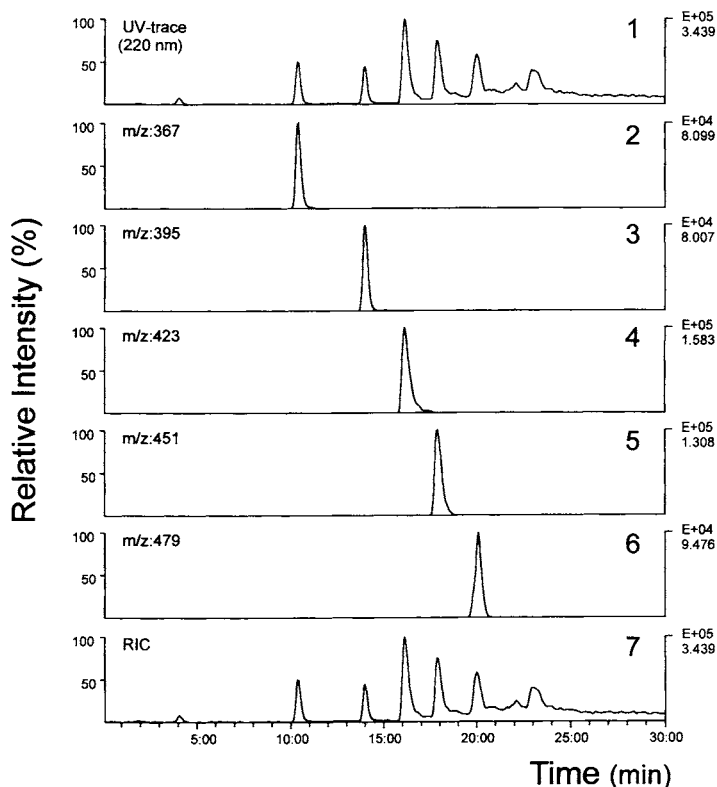
Fig. 19.36. ESI-LC-MS(+) total ion current chromatogram and selected mass traces of an industrial blend of sulfobetaines (C_nH_{2n+1}–C(O)–NH–(CH$_2$)$_3$–N$^{\oplus}$((CH$_3$)$_2$)–CH$_2$–CH(OH)–CH$_2$–SO$_3^-$) ionized as [M + H]$^+$ ions [142].

index (MBAS) or determination of bismut-active or disulfine-blue-active substances [28,29,30] in complex matrices that these methods give rise to problems. After primary degradation the metabolites of surfactants can no longer be detected in this way because the slight modification of the anthropogenic precursor molecules of the surfactant mixture prevents the detection. Therefore the results of surfactant analysis carried out in the past have to be estimated critically. False positive as well as false negative surfactant concentrations had been determined.

Today surfactants as well as their metabolites (primary degradation products) are detectable by the substance-specific determination method applying MS in combination with different ionization techniques [53,59,65,101,105,143,144]. However, if bond cleavage appears in the biochemical degradation process of non-ionic surfactants of the alkylpolyglycolether type, two metabolite types will be generated. The alkane moiety will be converted e.g. into a carboxylic acid. The polyether chain will be converted to polyether molecules. The sensitivity for both types of metabolites is quite different. The polyethers can be detected with excellent sensitivity whereas the TSP, APCI and ESI interfaces fail in the ionization of more or less unpolar compounds like alkanes, alkanols and even long-chain fatty acids.

The development of biochemical degradation products is easy, if degradation testing is carried out under controlled conditions like lab-scale waste water treatment plants using radioactive or stable isotopes. Especially radiolabeling allows tracking of the molecule to its ultimate fate. If real samples of treatment plant effluents are analyzed by MS, series of equally spaced signals with characteristic sequence like $\Delta m/z = 44, 58, 14$ or 28 may also indicate the development of metabolites in the waste water treatment process if these compounds cannot be identified as surfactants.

PEG and PPG as metabolites of the surfactants of the alkylpolyethylenglycol- and - polypropylenglycolether type [138] are normally found in the methanol eluate of RP-C_{18} SPE material after selective elution or in samples concentrated by freeze-drying after SPE [59]. Using ammonium acetate these degradation products can be identified in the form of their $[M + NH_4]^+$ ions, recognizable by their equidistant signals with $\Delta m/z = 44$ for the ions of PEG ($HO–(CH_2CH_2O)_n–H$) and 58 for the ions of PPG ($HO–(CH(CH_3)CH_2O)_n–H$). However, these compounds were also biodegraded resulting in carboxylated ($HO–(CH_2CH_2O)_{n-1}–COOH$) and di-carboxylated polyethers ($HOOC–(CH_2CH_2O)_{n-2}–COOH$) [138] as well as aldehyde compounds ($HO–(CH(CH_3)CH_2O)_{n-1}–CH(CH_3)CHO$) [79].

With the improvement of the MS equipment during the last years not only polyethers as metabolites of non-ionic surfactants but also carboxylates of non-ionics as well as of other surfactant groups like LAS, NPEO could be isolated and determined [53,59,65,101,105, 135,143,144]. Even aldehyde compounds [18,59,103], carboxylates of fluorine-containing non-ionics [17,92] and chemical reaction products of metabolites formed by bromine substitution during the drinking water treatment process and detected by FAB were reported [20].

19.3.5.1 PEG and PPG as metabolites of non-ionics

The analyses of surface water samples of the Saale river and the effluents of a WWTP discharging into the Saale river and a foam sample collected along the river [81] were done. The objectives were the qualitative and quantitative determination of surfactants and their potential metabolites PEG or PPG (cf. Sections 19.3.1, 19.3.2 and 19.5.1.1 or 19.5.1.2 and 19.5.1.5). After selective elution [18,59,92] the pollutants in the ether (PPG) and methanol (PEG) fractions were ionized in the ESI(+) mode applying ammonium acetate for ionization support.

PEG as a metabolite of alkylpolyethers with equally spaced ions ($\Delta m/z$ 44) at m/z 256 to m/z 740 was observed in the waste water effluents [81]. PPG, a biochemically more stable compound and a metabolite, too, was not only observed in the effluent of a waste water treatment plant, but also in the Saale river. These polypropyleneglycolether molecules were identified by their characteristic equally spaced ions at m/z 442, 500, 558 and 616 and in waste water samples from 326–732 ($\Delta m/z$ 58) [40].

The separation efficiency on a RP-C_{18} column for both mixtures of metabolites, PEG and PPG, was excellent. The total ion current traces as well as selected mass traces using ESI(+) ionization are presented in Fig. 19.37 [40].

After selective elution of PPG in the ether and PEG in the methanol fractions of the effluent SPEs of the WWTP of the city of Thessaloniki, Greece, these metabolites could be ionized by FIA-MS in the ESI(+) mode applying ammonium acetate for ionization
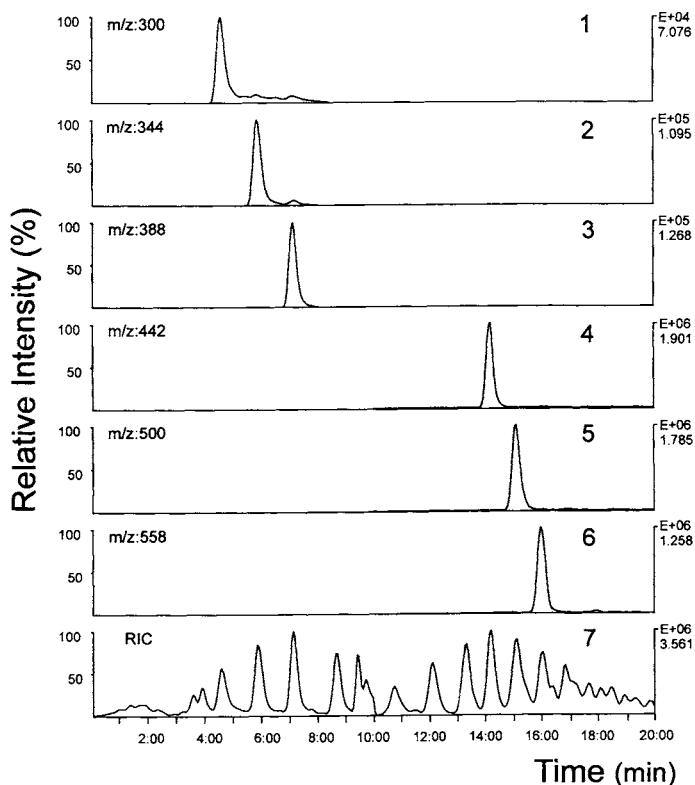
Fig. 19.37. ESI-LC-MS(+) total ion current chromatogram and selected mass traces of a waste water effluent containing PEG and PPG after C_{18}-SPE and selective elution ionized as $[M + NH_4]^+$ ions [40].

support [79]. Additionally PEG was also present the influent originating from anthropogenic discharges. However, the concentration of PEG in the effluent was many-fold originating from biochemical degradation of non-ionics.

19.3.5.2 Carboxylated PEGs and carbonylic PPG compounds

The biochemical oxidation products, carboxylates of PEG, as secondary metabolites of the primary metabolites (PEG) of non-ionic surfactants of the alkylethoxylate and alkylphenolethoxylate type were detected in tannery waste waters. These mono- (MCPEG) and dicarboxylate polyethoxylate glycols (DCPEG) can be confirmed by their ions at m/z 76 + $(44)_n$ ($[H-(OCH_2CH_2)_nO-CH_2-COOH + H]^+$) and 134 + $(44)_n$ ($[HOOC-CH_2-(OCH_2CH_2)_nO-CH_2-COOH + H]^+$), respectively using APCI-FIA-MS(+) [99].

The analysis of metabolites of non-ionic surfactants of alkylethoxylate type in waste water, surface water, seawater and groundwater samples was carried out using ESI-LC-MS(+). The metabolites derivatized as methyl esters and detected after a separation on a RP-C_{18} column were PEG, mono-carboxylated PEGs (MCPEG) and di-carboxylated PEGs (DCPEG), extracted before by SPE using graphitized carbon black (GCB) as
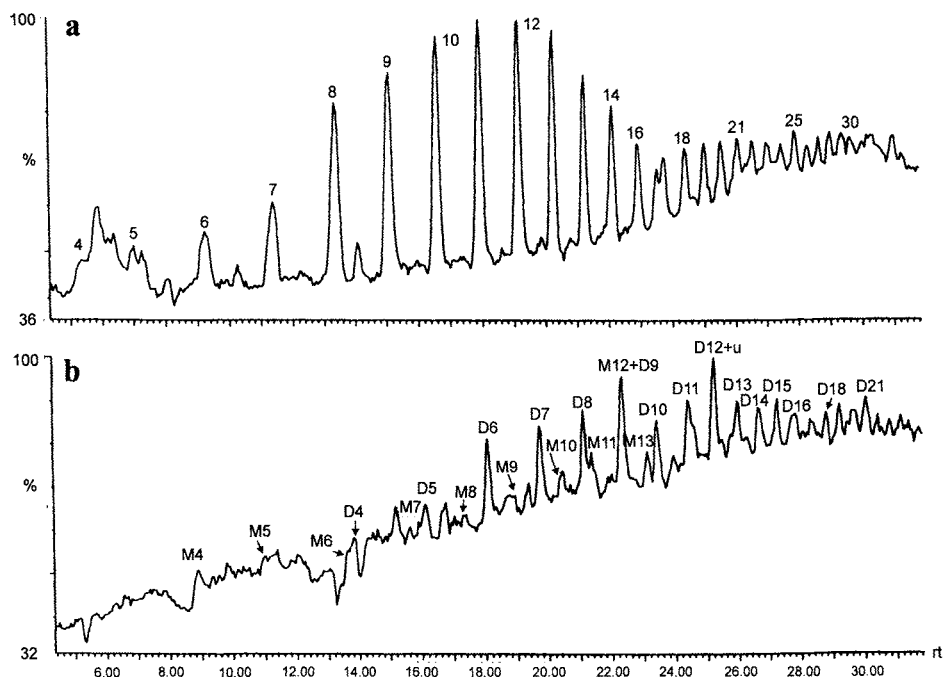
Fig. 19.38. ESI-LC-MS(+) total ion current chromatograms of carboxylated PEGs from waste water (a) influent and (b) effluent. Concentration by GCB-SPE and derivatized as methyl esters prior to ionization. Reproduced with permission from [86]. © 1997 by American Chemical Society.

solid phase material. For extraction neutral and acidic conditions were applied [86,143]. The full-scan total ion mass traces of these extracts in form of their methyl esters were presented in Fig. 19.38. Quantification using commercially available MCPEG and DCPEG homologues combined with recovery studies (cf. Section 19.5.1.5) allow statements about the concentrations in the media analyzed [86].

A metabolite of PPG, never verified before, could be recognized using APCI-FIA-MS and identified by FIA-MS-MS(+) (see Section 19.4.5). By analogy with biochemical degradation of alkyl polypropylene glycol ethers [103] this degradation of PPG resulted in short chain aldehyde homologues with the general formula $HO-(CH(CH_3)-CH_2-O)_x-CH(CH_3)-CHO$ ($x = 3$ or 4), presenting $[M + NH_4]^+$ ions at m/z 266 or 324, respectively [79].

19.3.5.3 Carboxylated perfluoroalkyl ethoxylate compounds

The non-ionic fluorine-containing polyethoxylated surfactant ($C_nF_{2n+1}-(CH_2-CH_2-O)_m-H$) has proved its extreme stability against chemical and physical attacks (cf. Section 19.3.1.1) [17], however, biochemical degradation was possible. In the waste water treatment process acidic metabolites ($C_nF_{2n+1}-(CH_2-CH_2-O)_{m-1}-CH_2-COOH$) had been formed. After storage of several years at a temperature $< -20°C$ the same mixture used for TSP examination before was ionized by ESI. Metabolites now were detected by ESI-

FIA-MS($+$) as $[M + NH_4]^+$ ions. In contrast to TSP($+$) ionization generating only ions at m/z 484 and 528 ($n = 6$; $m = 2$ or 3), ESI($+$) produced a series of equally spaced ions starting at 484 up to 834 ($n = 6$; $m = 10$) [17,59,92]. This result shows the improved ionization efficiency of ESI for polar compounds compared to TSP interfacing [104].

19.3.5.4 Mono and dicarboxylated alkylphenolethoxy compounds

Nonylphenolethoxylates (NPEO) used as non-ionic surfactant are produced synthetically in large-scale production processes with a complex mixture of isomeric and homologue compounds. The general formula $C_9H_{19}-C_6H_4-O-(CH_2-CH_2-O)_m-H$ does not show the real branching of the alkyl chain, which determines the biodegradability of these isomeric and homologue compounds in the waste water treatment process. The increasing estrogenic effect of the metabolites after polyether cleavage is known [7–9]. Under anaerobic conditions nonylphenol (NP; $C_9H_{19}-C_6H_4-OH$) is the main degradation product, concentrated in the sludge. For aerobic transformation carboxylation of both, the alkyl chain and the polyether chain, was postulated [145].

Di Corcia et al. [146] proved this postulate now by carrying out degradation experiments in laboratory scale, which resulted in either nonylphenolethercarboxylates (NPEC; $C_9H_{19}-C_6H_4-O-(CH_2-CH_2-O)_{m-1}-CH_2-COOH$), alkyl chain carboxylated NPEOs (CNPEO; $HOOC-(C_nH_{2n})_x-C_6H_4-O-(CH_2-CH_2-O)_m-H$) or compounds carboxylated in both positions, i.e. in the alkyl chain and in the polyether chain (CNPEC; $HOOC-(C_nH_{2n})_x-C_6H_4-O-(CH_2-CH_2-O)_{m-1}-CH_2-COOH$). Additionally these compounds could be generated in the mechanical-biological WWTP [146]. Compounds could be detected by ESI-MS($+$) after LC separation of Carbograph SPE eluates on RP-C_{18} material (see Fig. 19.39). Identification (see Section 19.4.2.6) and quantification (see Section 19.5.1.5) were
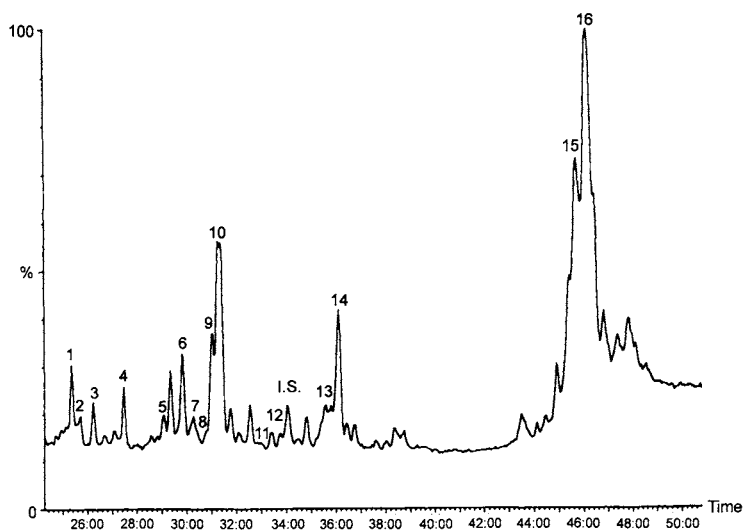
Fig. 19.39. ESI-LC-MS($+$) total ion current chromatogram of acidic extract of NPEO biodegradation test solution containing carboxylated NPEOs. Concentration by Carbograph-SPE. Reproduced with permission from [146]. © 1998 by American Chemical Society.

carried out in the form of their methylesters after derivatization of the acidic compounds. The dicarboxylated compounds ($CNPE_1C$) were recognized as extremely recalcitrant intermediates in the degradation process [146].

Metabolites of non-ionic surfactants of the alkylphenolethoxylate type were detected in surface water samples containing agricultural runoff. The compounds were concentrated by C_8-SPE. For elution of the cartridges methanol/water (95/5; v/v) was used prior to further concentration and the following FIA-APCI-MS(+) determination of the surfactants contained. Besides metabolites, e.g. carboxylates of nonylphenol-ethoxylates (NPEC) and the metabolite PEG, NPEO were contained in this fraction (cf. Section 19.3.1.3) [105]. The NPEO metabolites detected were carboxylated at the terminal polyether group of the polyether chain.

The short chain NPE_0C (C_9H_{19}–C_6H_4–O–CH_2–COOH) synthetically prepared for standard comparison purposes, could be ionized by APCI-FIA-MS in the positive and negative mode. In the presence of ammonium acetate APCI(+) resulted in $[M + NH_4]^+$ ions at m/z 296, whereas negative ionization generated the prominent $[M - H]^-$ ion besides $[M - H + acetate]^-$ and the dimeric ions $[2M–H]^-$ at m/z 277, 337 or 555, respectively [72].

Treated sewage water contained metabolites of NPEOs as confirmed by ESI-LC-MS(+) in the SIM mode. The ions of NPEO homologues carboxylated in the polyether chain were generated as $[M + H]^+$ ions equally spaced with $\Delta m/z$ 44 covering the range from m/z 367 (NPE_3C) to 675 ($NPE_{10}C$). These ions belonged to the carboxylated NPEOs with 3–10 EO units in the polyether chains [32].

19.3.5.5 Carboxylated metabolites of LAS

Short-chain intermediates carboxylated in the alkyl chain as potential metabolites of LAS were synthesized as standard compounds [54,147] and studied by ESI-LC-MS(−) [72]. The structural formulae of these potential metabolites, so-called SPCs, were shown in Fig. 19.40 I–III. The general formula of the calcium salts (CPCs) with the varying number of links in the alkyl chain were presented in Fig. 19.40 IV. These compounds, not yet confirmed in environmental samples, could be separated using ion-pairing chromatography on a RP-C_{18} column as shown in the mass traces in Fig. 19.41,3–5 and in the total ion mass trace in Fig. 19.41,6. For ion-pairing purpose mono ethyl ammonium acetate was applied. ESI(−) generates $[M - 1]^-$ ions at m/z 215, 229, 243 and 257 with a reduced sensitivity compared to the LAS standard added prior to separation and eluting from 27–31 min [72]. Methanolic solutions of the dimeric calcium salts of the CPCs in Fig. 19.40 IV were applied for ESI-FIA-MS(−) examinations. Salts dissociated in the presence of water and the anions resulted in signals at m/z 215, 229, 243 and 257. In parallel ionization of the SPCs resulted in ions at m/z 229 and 243. Separation efficiency in the traces 2–6 in Fig. 19.41 was poor compared to the UV-trace presented in Fig. 19.41 ,1. This UV-trace proved excellent separation results because tetrabutyl ammonium hydrogensulfate (TBAHSO₄) was applied for ion-pairing separation. However, this ion-pairing reagent is not amenable to ESI-LC-MS because of problems with the dramatic decrease of the signal to noise ratio (S/N) under these conditions [72].

Long-chain intermediates carboxylated in the alkyl chain as metabolites of LAS were studied at several sampling points in a salt marsh of the Bay of Cadiz (Spain) by ESI-LC-MS(−) [53] applying RP-C_{18} chromatography under ion-pairing conditions (methyl
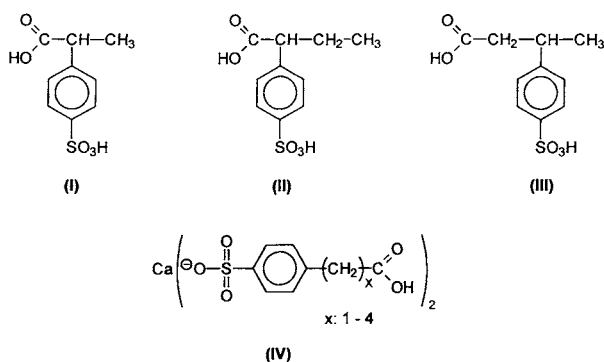
Fig. 19.40. I–IV. (I–III) Structural formulars of potential LAS metabolites carboxylated in the alkyl chain; (IV) Calcium salts of carboxylated sulfonic acids.
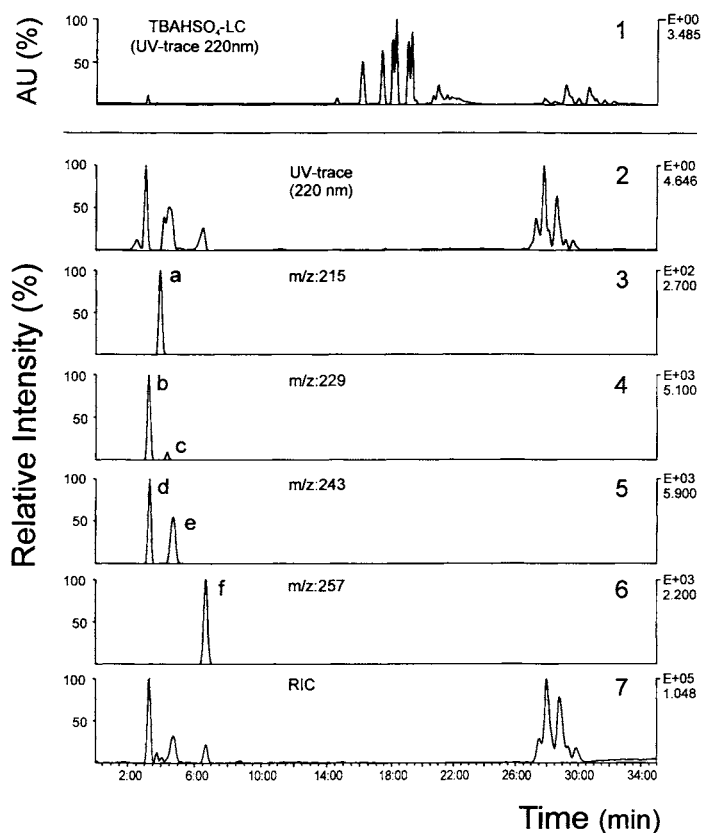
Fig. 19.41. ESI-LC-MS(–) total ion current chromatogram (RIC; 7) and selected mass traces (3-6) and UV trace 220 nm (2) of synthetic LAS-metabolites carboxylated in the alkyl chain separated on RP-C_{18} applying ion-pairing LC using ethyl ammonium acetate. UV trace 220 nm of LC separation on RP-C_{18} applying ion-pairing LC using tetrabutyl ammonium hydrogensulfate is presented in (1) [72].

triethylammonium acetate). After acidic extraction on C_{18}/SAX materials from the water and interstitial water samples the presence of LAS homologues (see Section 19.3.2.1) could be confirmed by LC with fluorescence and MS detection. As metabolites of LAS carboxylic degradation products $CH_3-(CH_2)_n-CH(C_6H_4SO_3H)-(CH_2)_x-COOH$ (sulfophenylcarboxylic acid; SPC; $n + x = 7$–13) with m/z 183 at elevated extraction voltages could be confirmed, too. The ESI-LC-MS(−) mass trace of the fragment ion 183 [53] is presented in Section 19.3.2.1, Fig. 19.19.

Applying APCI and ESI for the monitoring of the biochemical degradation process of WWTP (city of Thessaloniki, Greece) the anionic LAS besides non-ionic surfactants was found in the influent and effluent extracts. The monitoring approach consisted of screening applying FIA- and substance specific analysis by LC-MS and -MS-MS in the negative mode [135]. The methanol fraction of the SPE materials C_{18} and LiChrolut EN from the influent contained anionic surfactants ionized by ESI-FIA-MS(−) as $[M − H]^-$ ions at m/z 297, 311, 325 and 339 originating from LAS. However, comparing the FIA-MS(−) overview spectra of influent and effluent differences could be recognized because of the variations in their ion pattern. The FIA-MS(−) influent spectrum contained only the ions cited, whereas the effluent spectrum contained besides ions characteristic for LAS new ions with a difference of $\Delta m/z \pm 2$ Dalton from LAS characteristic ions (cf. Section 19.3.2, Fig. 19.20). No further information was provided about the ions at m/z 295, 309, 323, 337 and at m/z 299, 313, 327, 341 by FIA-MS(−). FIA-MS(−) and FIA-MS-MS(−) failed in the characterization and identification of the compounds contained in the effluent [135].

The methanol eluate of the effluent SPE was separated by RP-C_{18} LC-MS in the ESI(−) mode applying mono ethyl amine acetate for ion-pairing purpose. The TIC is presented in Fig. 19.42 together with the selected mass traces of the most abundant ions at m/z 311 and 325 and from the series of equally spaced ions with $\Delta m/z \pm 2$, i.e. ions at m/z 313 and 327 or 309 and 323. Besides the selected mass traces the UV trace 220 nm is enclosed in the figure. LAS was added as standard prior to separation. The result of the LC separation unequivocally allows the conclusion that all compounds ionized by FIA-MS(−) in the methanol fraction of the effluent were metabolites of LAS (retention time <31 min) (see Sections 19.3.5.5 or 19.4.5.2). The LAS spiked in the sample was separated with a retention time >32 min [135].

The compounds with ions at m/z 299, 313, 327 and 341 detected here seemed to be identical with compounds found in the Bay of Cadiz, i.e. these compounds should be long-chain carboxylic metabolites of LAS, so-called SPCs (sulfophenylcarboxylic acids), with the general formula $CH_3-(CH_2)_n-CH(C_6H_4SO_3H)-(CH_2)_x-COOH$ [53] (cf. Section 19.4.5.2).

19.3.5.6 Biogenic surfactants

Polyglucosides as environmentally friendly surfactants originating from polysaccharides and fatty alcohols today are produced and applied in increasing quantities (cf. Section 19.3.1). These anthropogenic compounds have biogenic parallels in the environment which are produced as surface-active metabolic products by bacteria. Two of these compound mixtures were isolated [148] and examined by API-FIA-MS, -LC-MS and MS-MS methods (see Section 19.4.1.6). At first the ESI-FIA-MS(+) spectrum of the rhamanolipids with the general structural formula shown in Fig. 19.43 contained three
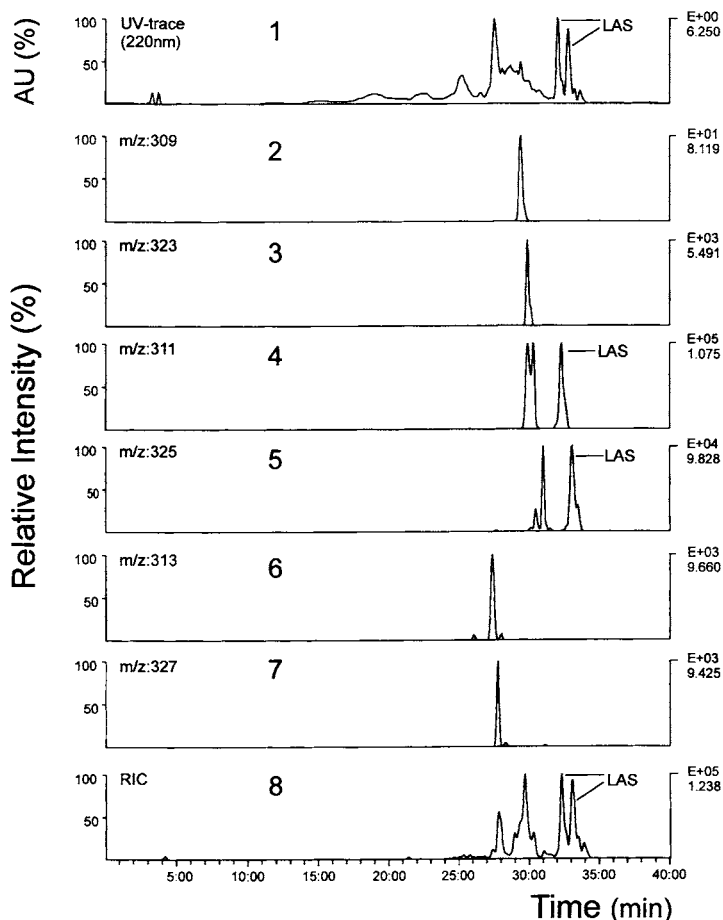
Fig. 19.42. ESI-LC-MS(−) total ion current chromatogram (RIC; 8) and selected mass traces (2–7) and UV trace 220 nm (1) of LAS-metabolites and spiked LAS standard separated on RP-C$_{18}$ applying ion-pairing LC using ethyl ammonium acetate [135].

dominating ions at m/z 376, 522 and 668, which belong to the $[M + NH_4]^+$ ions of these rhamanolipides. In parallel $[M + Na]^+$ ions, equally spaced with $\Delta m/z$ 5 to the ammonia adduct ions (Fig. 19.43) can be observed. The ions contain different numbers of sugar moieties resulting in different ions of the homologues at m/z 376, 522 and 668, equally spaced with $\Delta m/z$ 146 because of the number of sugar moieties [80].

The behavior of this compound mixture under RP-C$_{18}$ LC conditions is similar to that of alkyl polyglucosides as presented in Fig. 19.7. With an increasing number of sugar moieties in the molecules the rhamanolipids showed an increasing hydrophilic tendency expressed by a decrease of the retention time [80].

In addition the cyclic sorphose lipids (s. inset in Fig. 19.44), isolated with high yields as metabolites from cultures of *Candida bombicola* [149], can be estimated as a potential biogenic surface-active compound. ESI-FIA-MS(+) examination of this mixture resulted
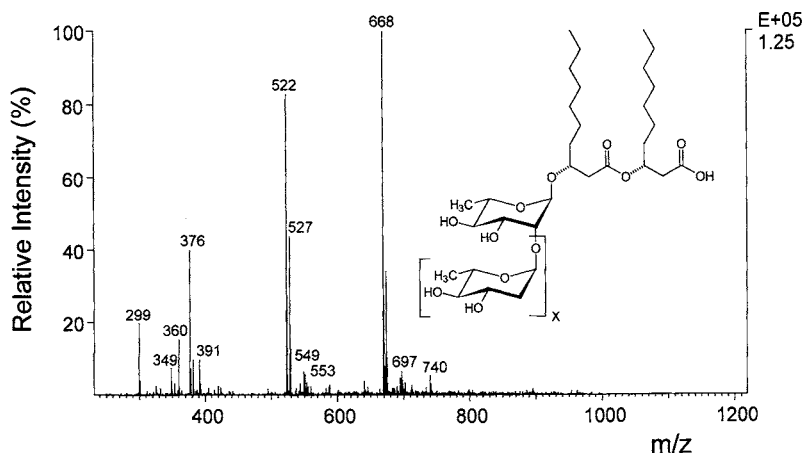
Fig. 19.43. ESI-FIA-MS(+) overview spectrum of biogenic rhamanolipid surfactants together with their general structural formula [80].

in three different ions at *m/z* 704, 706 and 708. The molecules of the homologue compounds contain two sugar moieties bridged by an ether bond as polar part of the molecule. A partly unsaturated alkyl chain as lipophilic part of the molecules contained one, two or three double bonds closing the cyclic structure to the sugars by ether bonds. Different molar masses which are due to a varying number of double bonds result in this series of ions with Δ*m/z* 2.

The chromatographic separation using ESI-LC-MS(+) under RP-C$_{18}$ LC conditions is shown in Fig. 19.44, presenting the TIC and the mass traces of the homologue ions [80].
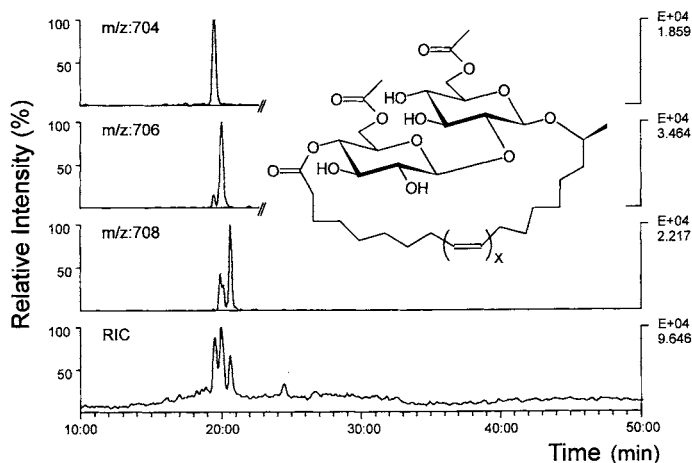
Fig. 19.44. ESI-LC-MS(+) overview spectrum of biogenic cyclic sorphose lipid surfactants together with their general structural formula [80].

19.4   IDENTIFICATION OF SURFACTANTS AND THEIR METABOLITES BY MS-MS USING FLOW INJECTION ANALYSIS (FIA-MS-MS) OR AFTER LC-SEPARATION (LC-MS-MS)

Under certain conditions the determination of surfactants, i.e. detection as well as identification, in water and soil is absolutely necessary [2,3,11]. Detection of surfactants is possible even in complex environmental samples e.g. from waste water, surface water and drinking water, however, the reliability of the results according to the standard methods [28,29] applied is not always satisfactory [30]. The different methods available for determination provide more or less convincing results. The first steps in surfactant analysis using substance group specific methods belong to these less convincing analytical techniques, whereas LC methods in combination even with unspecific detector systems nowadays had improved the analytical possibilities. However, the state of the art for getting more detailed information about surfactants in complex matrices can only be substance-specific analytical methods, since only they allow unequivocal identification of surfactants and their metabolites.

Applying MS using FIA or LC first steps towards substance-specific determination were done [59,65,143]. New types of mass specs in easy-to-use bench-top design are less expensive than triple quad instruments, but even more effective than the MS-MS machines to promote this development in future. Structural information essential for identification is provided by this bench-top equipment because of their MS^n options. The disadvantage of soft ionization applying thermospray, desorption or API-methods in combination with mass specs unsuited for MS-MS (cf. Section 19.1) will continuously decrease with the progress in the development of this modern mass spectrometric equipment.

APCI- or ESI (ion spray) as very soft ionization techniques produce adduct- ($[M + X]^+$) or molecular ions ($[M + 1]^+$ or $[M]^+$ in the presence of nitrogen) in the positive mode. The corresponding negative $[M - 1]^-$ ions or adduct ions such as $[M + acetate]^-$ are generated in the negative mode, as found in TSP ionization, too [59]. By using FIA or LC in combination with MS the information of the molar mass is received, but no fragments are produced which could provide structural information. Due to the large number of existing surfactants with all their isomers and homologues, the conclusions in identification drawn from MS examinations using soft ionization techniques like API, TSP [59] or FAB [65] have to be assessed very critically if no MS-MS was applied. The appearance of signal series and ion clusters with equidistant peaks such as $\Delta m/z$ 44 or 58 for surfactants or their metabolites with PEG- or PPG units in the molecule does not allow identification or characterization of such compounds as surfactants. In order to get the structural information, fragmentation of the parent ions by generation of structure-specific daughter (product) ions, is essential [73,74]. Some years ago, with the exception of special techniques like 'discharge-on mode', this could only be carried out by MS-MS if soft ionization techniques on commercially available mass specs were applied. Today collisionally induced dissociation (CID) in the MS-MS mode up to MS^n even on bench-top machines is used for the generation of structure-specific fragment ions (daughter ions), too. In parallel, the unequivocal identification of compounds such as surfactants, i.e. the exact knowledge of the structure, today can also be received by skimmer- or source CID under variation of the collision energy even on none-MS-MS machines [53,146]. Only the knowledge of the structure then allows statements on the effect of these compounds on

the environment, whereas the information on the molar mass is not sufficient for this purpose. As many surfactants detected in this way they have identical molar masses but isomeric structures, and therefore their ecotoxicological relevance can only be assessed if determinating specific structure elements are known [2,3]. If daughter ion spectra can be generated by the different methods mentioned here and the parent ion fragmented was originating from one compound, the identification of the compound is possible. For this purpose either the interpretation of the fragment spectra is necessary, or, if a daughter ion library containing surfactants or their metabolites (polar water constituents) is available, the compounds can be identified successfully by the computer-aided comparison of daughter ion spectra [15,18,78,81,92,150]. From our own experiences with TSP or API-interfaces on a TSQ applied for the generation of daughter ions by CID, the daughter ion spectra only show marginal variations if different interfaces are used if the same mass spec was used. This behavior was expected [59]. Prerequisite was of course that the same parent ions under the same CID conditions like collision energy or collision gas and its pressure were examined. In the past compounds not ionizable as intact ions like AES (cf. Section 19.3.2.4) or quats (cf. Section 19.3.3) could now be determined in the same form in which they really existed. Here new results can be expected.

To identify a compound by spectroscopic methods contained in diluted form in a mixture, it is above all necessary to get a concentrate of the compound to be determined. Moreover, the compound should be as pure as possible, i.e. the compound has to be concentrated and separated from other matrix constituents prior to spectrometric examination. And last but not least, the spectrometric detection and identification method should provide substance-specific information about the compounds to be examined.

Besides other methods (cf. Section 19.2.1) especially SPE using different materials is an excellent method to concentrate surfactants and their biochemical degradation products from environmental samples like all kind of waters.

MS would be the most appropriate method for substance-specific detection and identification.

Two different principles can be used in combination with MS to get pure compounds prior to MS determination.

Firstly, chromatographic separation methods like GC or LC belong to the appropriate techniques to separate either the volatile or the polar, non-volatile analytes from matrix compounds. In parallel to these separation techniques the compounds to be determined will also be focused on the separation column during the chromatographic pretreatment step which improves the sensitivity of detection.

Normally GC separation provides sharp signals, whereas LC separation leads to broad signals at the detector applied. Since the compounds to be analyzed are polar, the polarity of the compounds determines the separation method. Therefore techniques using liquids as mobile phases are able to solve this problem, and consequently LC is the separation method of choice for the separation of polar compounds like surfactants or their metabolites. Techniques like CZE with its improved separation compared to LC can also be applied. By contrast detection and identification by GC-MS demand a derivatization step prior to GC separation to make polar compounds volatile and applicable to this separation technique. All problems arising from this pretreatment step like discrimination because of an incompatible derivatization reagent were pointed out and discussed before.

Secondly, mixture analysis by-passing the analytical column (FIA-MS-MS), as

mentioned before, enables in many cases quick detection and identification of part of the compounds. This procedure was described in detail in the literature [62,73,74], and therefore is very briefly presented here: applying flow injection the sample is injected into the mobile phase pumped into the mass spec via an appropriate interface (FIA-MS). The mass spectrum (overview spectrum [59]) resulting from this operation can be regarded as a survey of API-active (APCI or ESI) components present in the sample. This is the first separation step, separating the compounds by their mass to charge ratio (m/z). In this case API ionization techniques fortunately are generating molecular or adduct ions with very little or without any fragmentation, i.e. only the information on the molar mass is provided by the soft ionization methods, APCI and ESI.

With both separation methods, LC as well as FIA-MS analysis, substance characteristic fragments are essential for identification. The procedures for identification using MS-MS are absolutely identical in LC- or FIA-MS analysis. This can be done after LC separation with MS detection or in flow injection MS mode by selecting ions of interest, using the mass filter of the tandem mass spectrometer. Ions to be determined then enter the collision cell of the mass spec. On their way to the detector the selected ions (parent ions) collide with collision gas atoms. These gas atoms, noble gas atoms (argon or xenon) under reduced pressure, generate fragment ions in cause of collision. The daughter (product) ions thus formed will then be detected according to the mass to charge ratio (m/z). The final result of this collision-induced dissociation (CID) step is the daughter ion mass spectrum characteristic for the parent (precursor) compound, if no isomeric compounds with the same m/z ratio were present in the mixture. Otherwise the development of mixed spectra from isomeric compounds could lead to misinterpretation and would prevent identification. Here LC separation or fractionation by selective SPE elution prior to FIA (cf. Section 19.2.2) can be successfully applied. In addition to the MS-MS methods applied on tandem mass specs, source or skimmer CID can also be used in the identification process for the generation of fragments. However, reproducibility has to be estimated critically, because it was found that even small changes in the flow rate may determine the fragmentation behavior. So the generation of daughter ions increased with reduced flow rates as confirmed in the separation of stearyl alcohol ethoxylates, providing more structural information. Fragmentation efficiency for PEG-esters was found to be higher than for PEG-ethers [68].

The daughter ion spectra generated by these alternative fragmentation techniques can be saved in libraries for comparison purposes. Like EI (electron impact) spectra libraries, daughter ion libraries generated by different CID methods allow the characterization of unknown compounds, too. However, the mass spec type applied for the generation of daughter ions conclusively determines the comparability of CID spectra, i.e. only spectra of identical mass specs, run under identical conditions, are comparable.

Both techniques have advantages and disadvantages as discussed before. Besides the generation of mixed spectra as a disadvantage in mixture analysis, the possibilty of ionization suppression reactions during the ionization of mixtures due to different proton affinities of the constituents cannot be neglected.

Although there are some important reasons for on-line LC-MS-MS compared to FIA-MS-MS, time-saving with FIA is fascinating. Using selective elution after SPE, it is tried to compensate as far as possible the disadvantages mentioned before (cf. Section 19.2.1). If selective elution fails, the mixture has to be separated by LC prior to MS-MS [81]. This

requires extensive work in advance [66] because the LC- as well as the CID conditions have to be optimized. Sometimes this procedure does not lead to the desired results. In surfactant analysis it may happen that surface-active compounds cover the surface of the analytical column. Only with a high share of organic solvents and special mixtures they can be completely removed [18,66]. Due to this fact retention time modifications occur during the LC-MS-MS course and may prevent any information, if the different CID windows are set too narrow. LC-MS-MS by source or skimmer CID will not be affected by this phenomenon. However, the question remains whether the results obtained with this technique are optimized results. The time-consuming and therefore expensive procedure in the on-line API-LC-MS-MS mode is the reason that only few results have been elaborated and published up to now using this procedure for the identification of surfactants and their metabolites.

19.4.1 Non-ionics

Non-ionic surfactants, especially PEG- and PPG derivatives (cf. Fig. 19.2), belong to those compounds which are most intensively studied by MS-MS in combination with FIA or LC in the extracts of waste water, surface water and drinking water.

Influents and effluents of waste water treatment plants may be highly loaded with non-ionic surfactants [59], as was demonstrated by FIA- and LC-MS analysis applying API interfaces (cf. Section 19.3.1). The eluates of waste water SPE using different materials like RP-C_{18}, LiChrolut EN, GCB were examined either by FIA-MS-MS doing mixture analysis or after LC separation in order to identify the alkylpolyglycolethers (C_nH_{2n+1}–O–(CH_2–CH_2–O)$_m$–H) or -propyleneglycolethers (C_nH_{2n+1}–O–($CH(CH_3)$–CH_2–O)$_m$–H). For this purpose daughter ion spectra of parent compounds forming series of equidistant ions with $\Delta m/z$ 44 or 58 in the FIA-MS spectra were generated by CID. The results were verified with the help of surfactant mixtures with known composition, produced in industrial scale, commercially available formulations or compounds received by biochemical degradation or targeted synthesis [59]. In addition mixture analysis applying parent ion scans like m/z 87, 89, 183 or 291 was used for the confirmation of carbonylic metabolites of non-ionic surfactants of the alkylpolyglycolether type [81], non-ionic surfactants of the alkylpolyglycolether type [81], LAS [96,135] or NPEO [81] in complex mixtures, respectively.

19.4.1.1 Alkylpolyglycolethers

Non-ionic surfactants are not only observed in drinking water, surface water or waste water and other environmental samples but also in things of daily use, such as bottle tops etc. However, they are also found in laboratory equipment like glass fiber filters [18,92] or in syringe filters [96]. Compounds of the alkylethoxylate type were detected qualitatively in the FIA-MS(+) mode originating from a single-use syringe filter used for sample filtration. The series of ions, forming a Gaussian curve with equally spaced signals ($\Delta m/z$ 44) belong to compounds with the general formula C_nH_{2n+1}–O(CH_2CH_2O)$_m$H. Under these conditions the ESI interface produced [M + NH_4]$^+$ ions. For identification of the compounds FIA-ESI-MS-MS(+) of the parent ion m/z 528 was applied. The ion submitted to CID was characterized by its daughter ions as a homologue compound of an

alkylethoxylate surfactant mixture with the formula $C_{10}H_{21}-O(CH_2CH_2O)_mH$ ($m = 8$) [96].

Standards of non-ionic surfactants of the polyether type were determined using APCI-FIA-MS(+) for screening purposes. According to the MS conditions applied fragmentation of the alkyl ethoxylates ($C_nH_{2n+1}-O(CH_2CH_2O)_mH$) with different alkyl and polyether chain lengths due to source CID effects could be observed. Alkyl chain as well as polyether chain fragments were generated and allowed identification of the alkylethoxylates [99]. Fragment ions at m/z 141, 169 and 271 were attributed to the aliphatic chains of decylic, lauric and tridecylic ethoxylates. Daughter ions between m/z 107 and 327 all equally spaced ($\Delta m/z$ 44), were consistent with polyether fragment ions originating from the alkylethoxylates. [99].

Compounds of the alkylethoxylate homologue type (Brij 78) are used as biological detergents and purification reagents. This compound mixture was detected by MS and identified by MS-MS using API-FIA because of their characteristic daughter ions at m/z 341 and 385 ($[CH_3(CH_2)_{17}(OCH_2CH_2)_n]^+$) [151]. Results of CID-MS-MS enabled the authors to identify these compounds by their daughter ion spectra using mixture analysis (FIA-MS-MS(+)).

Alkylpolyglycolether surfactants and their metabolites (cf. Section 19.4.5.1) were found as dominating polar organic pollutants in the Elbe and Saale rivers (East Germany). The non-ionic surfactants were identified by FIA-MS-MS(+) in combination with APCI or ESI interface [81,88] after C_{18}-SPE prior to selective elution by diethylether [81]. Non-ionics of the alkylethoxylate type with $[M + NH_4]^+$ ions at m/z 350–570 ($\Delta m/z$ 44) are to be classified as surfactant of the general formula $C_{13}H_{27}-O(CH_2CH_2O)_mH$ ($m = 3$–7) in the surface water samples. Because of the complexity of the overview spectra mixture analysis by FIA-MS-MS(+) was applied using diagnostic parent scans like m/z 89 for aliphatic non-ionic surfactants of polyether type [81].

Non-ionic surfactants were also determined in samples of a WWTP effluent, surface water and foam resulting from an overflow drop in a tributary of the Elbe river [40,88]. In APCI-FIA- as well as -LC-MS(+) analysis non-ionic surfactants have been characterized because of their equidistant signals. Since this pattern appearing in series of equal-spaced signals may also occur with anionic as well as cationic surfactants (cf. Sections 19.3.2 and 19.3.3), MS-MS either generating daughter ion or parent ion spectra was necessary to confirm these estimations. MS-MS(+) results proved this characterization as alkylethoxylates ($C_nH_{2n+1}-O(CH_2CH_2O)_mH$) with different alkyl and polyether chain lengths [40,88].

The characterizations done by FIA-MS of the non-ionic surfactants found in influent and effluent extracts of WWTP of Thessaloniki, Greece, were examined by FIA-MS-MS using APCI and ESI in the positive and negative mode (cf. Section 19.3.1). With the help of the daughter ions of the ion m/z 394 from the influent extract this compound could be unequivocally identified as alkylpolyethylenglycolether with the formula $C_{13}H_{27}-O-(CH_2-CH_2-O)_4-H$ [79]. The daughter ions m/z 45, 89, 133 and 175, known as polyether fragments of the PEG chain, and the alkyl fragments m/z 57, 71, 85, 99, 113 and 127 are characteristic for these alkylpolyethylenglycolethers (Fig. 19.45) [59].

More CID spectra of selected ions such as m/z 482 from the SPE of the methanol eluate of the influent indicated the presence of the alkylpolyglycolether homologue with the formula $C_{13}H_{27}-O-(CH_2-CH_2-O)_6-H$. The ions of these non-ionic surfactants could be observed as $[M + NH_4]^+$ ions exhibiting the characteristic behavior of this compound
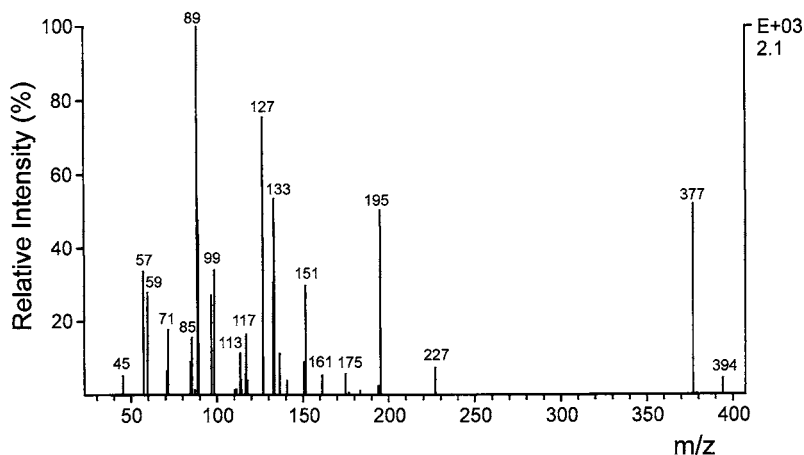
Fig. 19.45. APCI-FIA-MS-MS(+) (CID) daughter ion mass spectrum of unknown parent ion with m/z 394 from C_{18}-SPE waste water extract of WWTP Thessaloniki [59].

group: formation of alkyl- and polyether fragments. In the effluent sampled corresponding to the retention time of the sewage only the non-degradable compounds and the metabolites of the surfactants were present [79].

The ionization of the fluorine-containing surfactant blend (C_nF_{2n+1}–(CH_2–CH_2–O)$_m$–H) using ESI(+) and APCI(+) ionization in the FIA-MS mode resulted in modified overview spectra compared to TSP-FIA-MS(+) spectra [69,92]. The partition of ions now varies favouring the short alkyl chain compounds. However, the behavior under ESI-FIA-MS-MS(+) mode was identical compared to the TSP spectrum generated by CID [17,59].

19.4.1.2 Alkylpolypropyleneglycolethers

The industrial blend of a non-ionic surfactant of the alkylpolypropyleneglycolether type (C_nH_{2n+1}–O–($CH(CH_3)$–CH_2–O)$_m$–H) was examined by APCI-FIA-MS-MS(+). Fragmentation behavior was identical with the results generated by TSP ionization [18,59]. Daughter ion spectra contain alkyl fragments at m/z 57, 99, 115, 157, 173 and 215 as well as the fragments of the PPG chain at m/z 59, 117 and 175 [104].

Examination of the influent and effluent extracts of the WWTP of Thessaloniki, Greece, applying APCI and ESI-FIA-MS-MS(+/−) confirmed results obtained by FIA-MS (cf. Section 19.3.1).

The daughter ion spectrum of the ion at m/z 510 from the ether eluate of the influent SPE unequivocally confirmed the presence of the alkylpolypropylenglycolether homologue with the formula C_9H_{19}–O($CH(CH_3)CH_2O$)$_6$–H [79]. The fragment ions with m/z 59, 117 and 175 had developed from PPG polyether chains (Fig. 19.45) [59]. The few alkyl fragments (m/z 85 and 127) in the CID spectrum of the alkylpolypropylenglykolether examined indicate a strongly branched alkyl chain [79].
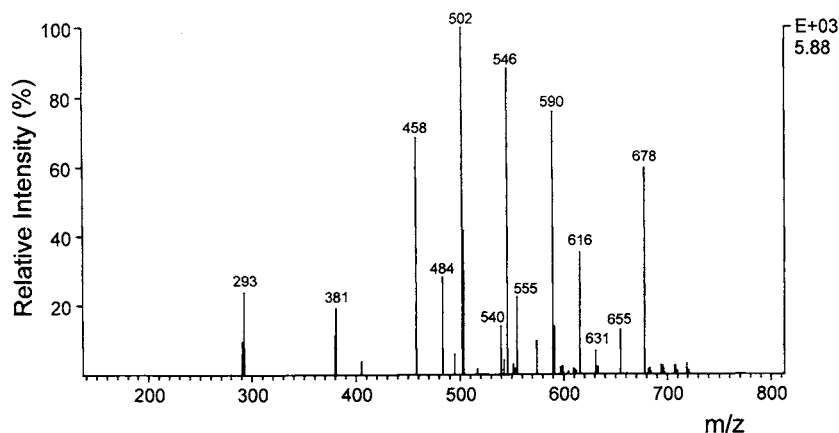
Fig. 19.46. APCI-FIA-MS-MS(+) (CID) parent ion mass spectrum of product ion with *m/z* 291 of C_{18}-SPE surface water extract (river Elbe) [81].

19.4.1.3 Alkylphenolpolyglycolethers

During qualitative and quantitative determination (cf. Sections 19.3.1 and 19.5.1.1) of the surfactant contents of surface water polluted by a WWTP effluent and foam resulting from an overflow drop of an Elbe tributary [81,88], complex mixtures of different surfactants were observed. To confirm the presence of nonylphenolethoxylates (NPEO; C_9H_{19}–C_6H_4–O–$(CH_2$–CH_2–O$)_m$–H) in the water samples the parent ion mass spectrum of *m/z* 291 was generated by MS-MS in the FIA-APCI(+) mode. Despite the fact that the NPEOs were present in very low concentrations in the water sample, the series of homologue compounds could be confirmed by the parent ion scan *m/z* 291 as demonstrated in Fig. 19.46 [81].

Besides other polar compounds NPEO homologues were determined by APCI-LC-MS-MS(+) in complex samples from the Elbe river recording the substance-characteristic ion at *m/z* 291 [88].

NPEOs in waste water samples were screened by using precursor ion scanning of *m/z* 121 and 133 and multiple reaction monitoring (MRM) in the API-FIA-MS-MS(+) mode. Under the sewage treatment plant (STP) conditions the parent scan (PS) of 121, characteristic for ethoxylates with one to four chain units (EO_1–EO_4), and the PS of 133, characteristic for EO_5–EO_{16}, demonstrated a preferential elimination of the EO_5–EO_{16} homologues of NPEO of Igepal CP-720 type. Since the PS alone was not characteristic for these compounds because of the interference with linear alcohol ethoxylates, monitoring 16 MRM transitions confirmed the results of PS [107]. Semiquantitative estimations (see Section 19.5) were possible demonstrating different removal rates of the homologues according to their PEG chain length.

Environmental water samples of the Tama and Meguro rivers in Japan were analyzed using ESI and APCI in the FIA mode after column separation and fractionation. First attempts were made to identify the compounds present in the water as octylphenolethoxylates (OPEO) by using APCI-MS-MS(+) in combination with a standard OPEO formulation. However, separation combined with UV-detection in the normal-phase mode

provided the information that the compounds present in the environmental sample were different from OPEO [110]. FIA-MS applied provided spectra containing equally spaced signals, but the intervals of $\Delta m/z$ 74 of the homologue ions were different from polyglycol ether ions as postulated in OPEO blends ($\Delta m/z$ 44). The authors speculated on the elemental composition of these compounds found in river water and effluents of WWTPs, with the result that persistent poly(glycidyl) monofluorooctylphenyl ethers were 'identified' because of the analytical information available. But no CID information was presented to support this hypothesis [110].

Nonylphenolethoxylates (NPEO) were examined using APCI-FIA-MS(+). Source CID(+) resulted in fragmentation exhibiting characteristic ions of alkylphenolethoxylates as presented in Fig. 19.3 (Section 19.3.1) besides alkyl and polyether fragment ions. In addition to the structural information by CID retention time and calculated compositions of the compounds ionized were used for characterization and identification of surfactants in tannery waste waters [99].

Octylphenolethoxylate homologues like Triton X-100 are used as biological detergent and purification reagent. This compound mixture was detected by MS and identified by MS-MS using API-FIA because of their characteristic daughter ions at m/z 233, 277 and 321 ($[C_8H_{17}C_6H_4(OCH_2CH_2)_n]^+$) as well as m/z 121 165 and 209 ($[C_3H_7C_6H_4(OCH_2CH_2)_nH + H]^+$) [151]. Results of CID-MS-MS spectra indicated that all Triton X-100 ions were shared by PEG, explaining the relative low abundance of Triton ions at m/z 233 and 277.

19.4.1.4 Fatty acid polyglycol esters

The blend of a non-ionic surfactant mixture polluted by precursor compounds from synthesis was examined for its content of fatty acid polyglycolesters. ESI-FIA-MS-MS(+) was used to prove the presence of these fatty acid polyglycolesters with the general formula $C_nH_{2n+1}-C(O)-O-(CH_2-CH_2-O)_m-H$. PEG and other side reaction products were confirmed besides these polyglycolester homologues. CID of the parent ion appearing at m/z 406 as a $[M + NH_4]^+$ ion allowed to confirm the compound with $n = 15$ and $m = 3$ belonging to a series of equally spaced ions ($\Delta m/z$ 44) because of their polyglycolether chains (PEG). The daughter ion spectrum generated contained only one single ion at m/z 283. This product ion was generated by fission of the PEG chain and ring closure resulting in a cyclic ion as shown in Fig. 19.47. The parent ion scan of 283 presents the compounds at m/z 362, 406, 450, 494, 538 and 582 belonging to this type of surfactant, covering compounds with $m = 2$–7 [104].

Compounds of the fatty acid polyglycolester type (Tween 40 and Myrj 40) are also used in biochemistry as purification reagents. Detection by MS and identification by MS-MS using API-FIA was applied using their characteristic daughter ions at m/z 283 and 327 ($[CH_3(CH_2)_{16}COO(CH_2CH_2O)_n]^+$; $n = 1$ or 2) [151]. Results of CID-MS-MS enabled the authors to identify these compounds together with alkylphenoletoxylate surfactant homologues by their daughter ion spectra using mixture analysis (FIA-MS-MS(+)).

19.4.1.5 Fatty acid mono and diethanolamides

Fatty acid diethanolamides ($C_nH_{2n+1}-C(O)-N(CH_2-CH_2-OH)_m$; $n = 9,11,13,15$;

m/z: 406   m/z: 283
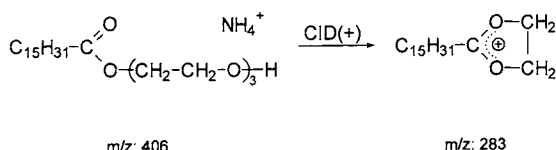
Fig. 19.47. Fragmentation behavior of fatty acid polyglycolesters under CID conditions applying ESI-FIA-MS-MS(+) [104].

$m = 2$) can be observed in the influents of waste water treatment plants. They can be easily identified by their characteristic TSP-MS-MS(+) spectra containing fragments with m/z 70, 88, 106 and 227 [18,59,66]. Under ESI-FIA-MS-MS(+) they show the same behavior as observed in the TSP mode. One important reaction in the CID process is the gas-phase rearrangement and elimination of the neutral 61 u ($[H_2N–CH_2–CH_2OH]$) generating the fragment ion m/z 227 if $n = 11$ [77,104], as shown in Fig. 19.48.

This behavior under CID conditions, loss of 61 u observed with diethanolamides, was also found with fatty acid monoethanolamides ($C_nH_{2n+1}–C(O)–N(H)–CH_2–CH_2–OH$; $n = 9,11,13,15$; m/z 216, 244, 272 and 300). However, fragmentation observed with diethanolamides like abstraction of water (loss of 18 u) was not possible but in addition fragments from the alkyl chain were found here at m/z 57, 71 and 85 applying ESI-FIA-MS-MS [104].

The commercial blend of the oleic acid diethanolamide ($CH_3–C_7H_{14}–CH=CH–C_7H_{14}–C(O)–N(CH_2–CH_2–OH)_2$) was ionized as $[M + H]^+$ ion at m/z 370 applying ESI-FIA-MS (+) (cf. Section 19.3.1.5), before this diethanolamide of an unsaturated fatty acid was examined by ESI-FIA-MS-MS(+). It showed a comparable behavior as saturated diethanolamides and resulted in fragments (m/z 88, 106 and (370-61=309)) [104], as observed from daughter ion spectra of fatty acid diethanolamides presented in Fig. 19.48 [18,59,66, 77].

Electrospray MS^n analysis leading to sequential product ions was used for the identification of an unknown surfactant in an extract of a shampoo formulation [77]. MS^4 experiments together with other spectral observations led to the hypothesis that the unknown compound was a N-(2-aminoethyl) fatty acid amide with the general formula $R–C(O)–NH(CH_2–CH_2–N)R'R''$. This could be confirmed by an authentic sample of the proposed laury amphomono acetate (LAMA) with the $[M + H]^+$ ion at 345 and $R' = –CH_2–CH_2–OH$ and $R'' = –CH_2–CH_2–COOH$. The fragmentation observed led to an intensive examination of amides. Only two of them, lauryl diethanol amide ($[M + H]^+$:

m/z = 227
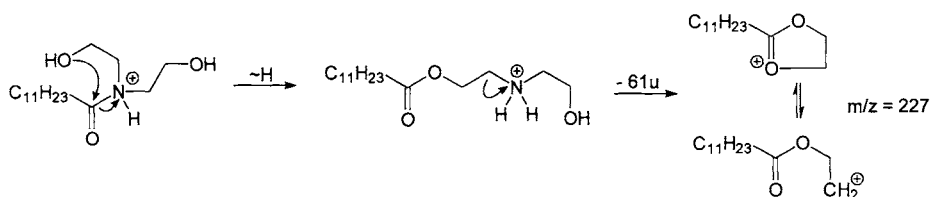
Fig. 19.48. Fragmentation behavior of fatty acid diethanol amides under CID conditions [77] applying ESI-FIA-MS-MS(+) [104].
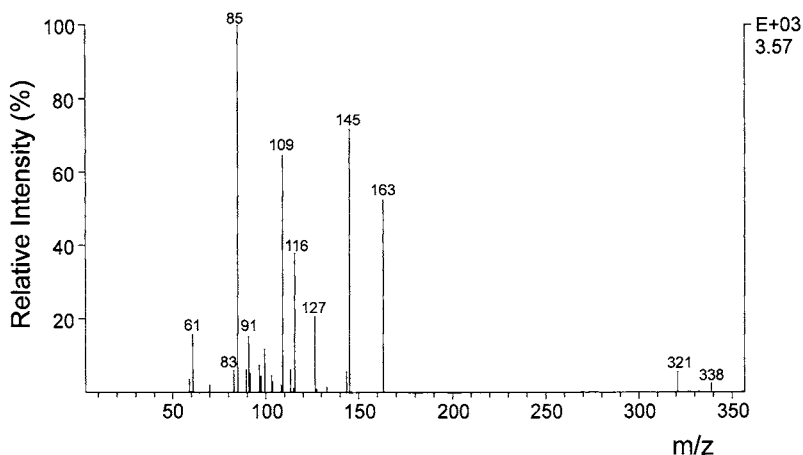
Fig. 19.49. APCI-FIA-MS-MS(+) (CID) daughter ion mass spectrum of alkyl monoglucoside homologue with m/z 338 from industrial blend [59].

288), a non-ionic surfactant [18] and laurylamido-β-propyl betaine ([M + H]$^+$: 343), showed a similar behavior as LAMA. MS-MS examination of surfactants on a TSQ mass spec led to the explanation that a gas-phase rearrangement prior to the fragmentation had happened or an isomer had been present. MS^3 experiments by source CID and MS-MS in the second quad of TSQ produced ions consistent with the ESI ion trap [77].

19.4.1.6 Alkylpolyglucosides

Alkylpolyglycosides (($C_6H_{11}O_6$)$_x$–R (x = 1–3; R = C_nH_{2n+1}; n = 8, 10, 12 and 14) (cf. Section 19.3.1.6) were examined by APCI-FIA-MS-MS in the negative and positive mode. Parent ions generated as [M + NH$_4$]$^+$ ions from the alkyl monoglucoside (AMG) homologues with m/z 310, 338, 366 and 394 were fragmented using CID. All these compounds belong to a series of AMGs differing by Δm/z 28 and produce the same characteristic fragment ions at m/z 85, 91, 99, 109, 115, 127, 145 and 163. Besides fragments from the sugar moieties some other daughter ions were produced by abstraction reactions of H$_2$O (Δm/z 18) from sugar moieties as recorded here (m/z 163 → 145 → 127). Therefore the daughter ion spectra of the AMG homologues can be very well used for identification purposes because of their identical pattern of signals. The daughter ion spectrum of the monoglucoside ($C_6H_{11}O_6$)$_1$–$C_{10}H_{21}$ is presented in Fig. 19.49 [69].

If the [M + NH$_4$]$^+$ ions contain more than one sugar moiety, no favoured successive sugar abstraction was found, moreover, the degradation by fragmentation happens in the alkyl chain, too [69].

19.4.1.7 Alkylglucamides

Alkylglucamides (C_nH_{2n+1}–C(O)N(CH$_3$)CH$_2$–(CH$_2$–OH)$_4$–CH$_2$OH) have not yet been determined in environmental water samples. The reason is that compounds may be easily biodegradable or are difficult to detect even by MS screening because of their rather
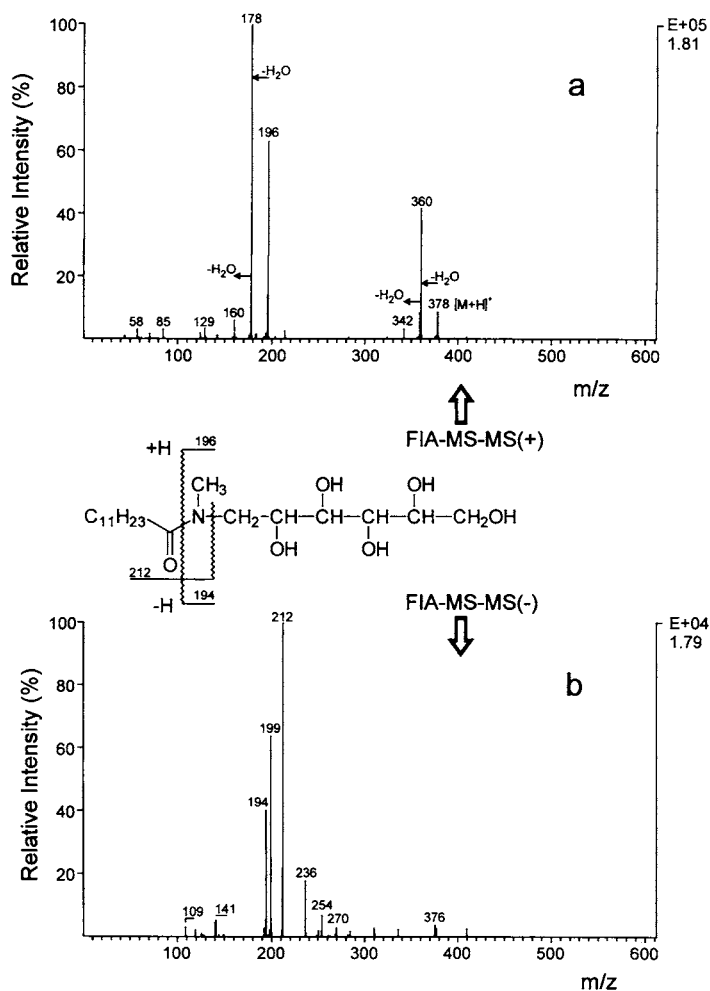
Fig. 19.50. (a) APCI-FIA-MS-MS(+) daughter ion mass spectrum of alkylglucosides homologue with m/z 378 ($[M + H]^+$) and (b) APCI-FIA-MS-MS(−) daughter ion mass spectrum of the same alkylglucamides homologue with m/z 377 ($[M − H]^-$) from industrial blend [69].

inconspicuous pattern of ions at m/z 378, 406 and 434 (cf. Section 19.3.1.7, Fig. 19.16). However, APCI-MS-MS(+) applied to all homologue parent ions of alkyl glucamides generates two significant daughter ions at m/z 178 and 196 (Fig. 19.50a). Using the parent ion scan of these ions, glucamides can be confirmed in this way. Using APCI-MS-MS in the negative mode fragment ions at different m/z ratios according to the alkyl chain length (C_{11}: m/z 199, 212) are dominating the CID spectrum (Fig. 19.50b). Additionally the ion observed at m/z 196 in the positive mode can now be observed as negative ion at m/z 194 [69]. Under CID conditions the positive ions at m/z 178 and 196 and the negative ion at m/z 194 can be used as diagnostic ions for the determination of alkylglucamides.

19.4.2 Anionics

Since non-ionic surfactants are detected as well as characterized in the positive mode, some anionics can be detected and characterized not only in the negative but also in the positive mode. However, anionics are predominantly or sometimes exclusively ionized in the negative mode using FIA- or LC-MS-MS. This selective ionization for detection or characterization of anionic surfactants from water extracts is very effective, in parallel compounds which are only ionized in positive mode are suppressed [59]. The overview spectra received by FIA-MS are very clear and free from disturbing 'matrix' components. As the matrix can be suppressed under these conditions, FIA- as well as LC-MS-MS results in the negative mode will not be disturbed.

Anionic surfactants, especially alkylbenzene sulfonates of the general formula C_nH_{2n+1}–(C_6H_4)–SO_3H, belong to those washing and cleaning agents which are most commonly used all over the world. However, data dealing with LAS identification by mass spectrometric methods are seldom found in literature. Since this type of molecule is equipped with a chromophoric phenylgroup, UV as well as fluorescence detection are used very often [32,53,152]. The negative CID spectra of LAS [M − 1]⁻ ions generated by ESI or APCI-ionization on MS-MS machines contain only few diagnostic signals. The number of fragments depends on the collision energy, collision gas pressure or extraction voltage used. The same phenomenon can be observed under source or skimmer CID conditions.

The fragmentation efficiency is independent of the alkyl chain length in LAS homologues covering C_{10} to C_{13} (*m/z* 297, 311, 325 and 339). The reason is that under CID conditions thermodynamically favoured ions with the structure $[CH_2=CH–(C_6H_4)–SO_3]^-$ at *m/z* 183 from the branched ABS will be formed. The LABS form the product ions (*m/z* 197) with the structural formula $[CH_2=C(CH_3)–(C_6H_4)–SO_3]^-$. This behavior found in FAB or TSP mode, too, is described in detail in the literature [59,65]. The signal intensity of parent ion and product ion(s) under CID conditions in tandem mass specs as well as under source or skimmer CID conditions varies according to the operational conditions cited before. Applying APCI or ESI, the generation of positive daughter ions of LAS or LABS was not possible.

The mixture analysis approach applying the parent ion scan was used to recognize LAS in the complex mixture of a waste water extract ionized by FIA-ESI-MS(−). The parent ion scan of *m/z* 183 was successfully applied for this purpose because the screening approach by FIA-MS failed. The ions at 283, 297, 311, 325 and 339, characteristic for LAS, could be recognized without problems because the parent scan 183 suppressed the matrix compounds present in the waste water extract [96].

19.4.2.1 Alkylsulfonates

Alkane- or alkylsulfonates (C_nH_{2n+1}–SO_3H) or their isomers, the secondary alkane sulfonates (SAS) with the modified formula $CH_3–(CH_2)_n–CH(SO_3H)–(CH_2)_x–CH_3$, can be ionized in the negative mode. FIA-MS-MS(−) applied to each homologue type using ESI or APCI results in daughter ion spectra which contain only one peak at *m/z* 80 ($[SO_3]^-$) besides the parent ion. The same result was found by generating negative daughter ions from alkenesulfonates. The parent ion scan of *m/z* 80 used in environmental

samples vice versa is an excellent diagnostic method for the recognition of these compounds [81,136].

19.4.2.1.1 Alkylbenzene sulfonates Anionic surfactants like alkylbenzene sulfonates $(C_nH_{2n+1}-(C_6H_4)-SO_3H)$ were ionized with high sensitivity and selectivity in the negative mode. Independent from the alkyl chain length (m/z 297, 311, 325 and 339) LAS or ABS show very few daughter ions under CID($-$) conditions. So daughter ions at m/z 183 ([CH_2=$CH-(C_6H_4)-SO_3$]$^-$) will be generated from the branched ABS whereas the LABS forms the product ion at m/z 197 ([CH_2=$C(CH_3)-(C_6H_4)-SO_3$]$^-$) [59,65,153]. Operating with an increased capillary exit voltage, besides the daughter ion at m/z 183 the [SO_3]$^-$ ion at m/z 80 could be observed applying ES($-$) ionization [131].

Using APCI-FIA-MS-MS($-$) LAS were identified in water samples from the Saale river by their characteristic daughter ion m/z 183 and retention time comparison in APCI-LC-MS($-$). The application of mixture analysis by generation of parent ions of 183 confirmed this analytical results of LAS determination in real environmental samples originating from river Elbe and its tributaries [88].

The short chain alkylbenzene sulfonates like ethyl or methyl benzene sulfonic acid (toluenesulfonic acid), however, exhibit daughter ions ([M $-$ H-64]$^-$) because of the loss of SO_2. Aliphatic surfactants like SAS, however, which also contain a sulfonate moiety [136], in addition produce ions at m/z 80 ([SO_3]$^-$).

In contrast to these results with toluenesulfonic acid presented before, the full scan ion chromatography MS-MS spectrum contained only the ion at m/z 80 ([SO_3]$^-$) [137].

Waste water (WW) samples were screened using FIA-ESI-MS($-$) [96]. However, the results were very poor because of matrix compounds present. Using MS-MS in the parent scan mode (precursors of m/z 183) allowed the identification of LAS present in WW. Ions at m/z 297, 311, 325 and 339, characteristic for LAS, can be clearly observed under these conditions [96].

19.4.2.1.2 Substituted benzene sulfonates Nitrobenzene sulfonate used as surfactant in industrial applications was detected by negative ESI-MS resulting in an ion at m/z 202. Its fragmentation behavior under elevated capillary exit voltage is shown in Fig. 19.51a resulting in the loss of SO_2 (m/z 138) or NO_2 (m/z 156) [131].

19.4.2.1.3 Alkylnaphthalene sulfonates Besides alkylbenzene sulfonates alkylnaphthalene sulfonates were also used as surfactants. Their identification is possible doing FIA-ESI-MS-MS($-$) by operating with an elevated capillary exit voltage [131]. According to the alkyl substituent (C_1-C_3) the daughter ions m/z 157,171 and 185 were found because of a loss of SO_2 besides the daughter ion at m/z 80 ([SO_3]$^-$), as shown in Fig. 19.51b.

19.4.2.1.4 Alkylethersulfates Alkylethersulfates (AES) $(C_nH_{2n+1}-O-(CH_2-CH_2-O)_x-SO_3H)$ up to now are very seldom determined in environmental water samples. The reasons are that these compounds are easily biodegradable, and that they are difficult to detect even by MS screening. TSP($-$) was found to be very insensitive whereas positive ionization leads to the destruction of the molecules resulting in the cleavage of the SO_3 moiety [59]. API methods are able to ionize these compounds, but varying results were received, as presented in Section 19.3.2.4. The most promising method for the detection of AES is ESI-FIA-MS ionization in the negative mode. Identification applying FIA-MS-
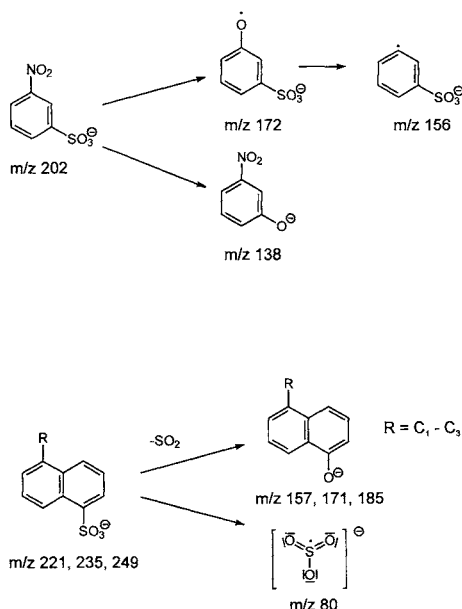
Fig. 19.51. (a) Fragmentation behavior of nitrobenzene sulfonate and (b) of alkylnaphthalene sulfonates under CID conditions applying ESI-FIA-MS-MS(−) [131].

MS(−) leads to a very simple daughter ion spectrum of all AES homologues containing the parent ion and one daughter ion with m/z 97 ([HSO_4]$^-$). The same behavior was found in the APCI-FIA-MS-MS(−) mode, however, the sensitivity was reduced.

Positive CID using APCI or ESI resulted in alkylethoxylate daughter ion spectra providing the information of the alkyl chain length and its branching. In this case alkyl ($\Delta m/z$ 14) as well as polyether fragments ($\Delta m/z$ 44) can be observed at m/z 57, 71, 85, 99, 113 and 127 or 45, 89, 133 and 177 from the [M + H − SO$_3$]$^+$ ions [70]. These results were observed when the parent ion 363 was examined by CID using MS-MS. This ion was originating from the AES molecule $C_{12}H_{25}$–O–(CH_2–CH_2–O)$_4$–SO_3H with the molar mass of 443 prior to the loss of SO$_3$ [70]. For the analysis of AES in environmental samples the negative parent ion scan of 97 was successfully applied. This is a good diagnostic method for confirming AES even in complex mixtures as found in the river Elbe [81].

19.4.2.2 Alkylsulfates

Alkylsulfates (AS) (C_nH_{2n+1}–O–SO_3H) were only used as surfactants for special applications, but they were also found as unreacted precursor compounds together with AES. Their identification in the presence of AES using FIA-MS-MS(−) may cause problems because the AS [M–1]$^-$ ions at m/z 265 and 293 of the C_{12} and C_{14} homologues both show only ions at m/z 97 ([HSO_4]$^-$), the same CID reaction products received from AES. Negative parent ion scan of 97 applying FIA vice versa will present AS as well as AES ions [136].

Synthetic mixtures of alkylsulfates (C_nH_{2n+1}–O–SO_3H; $n = 8$, 10, 12, 14 and 18) were

$$R-O-(CH_2-CH_2-O)_n-CH_2-C \overset{O}{\underset{OH}{\diagdown}} \xrightarrow{\text{API-CID(-)}} \overset{H_2C}{\underset{H_2C}{\diagdown}} \overset{\oplus}{\underset{}{O}}-CH=C \overset{O^{\ominus}}{\underset{O^{\ominus}}{\diagdown}}$$
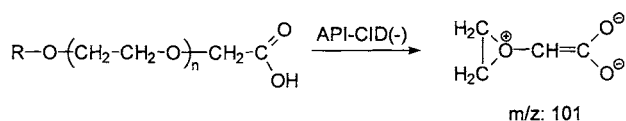
m/z: 101

Fig. 19.52. Structure of the specific daughter ion *m/z* 101 generated from alkylethercarboxylates under CID($-$) conditions [136].

separated and detected in the SIM ESI($-$) mode. MS-MS data of alkylsulfates were presented containing both ions, a $[SO_3]^-$ ion at *m/z* 80 with low intensity and the dominating $[HSO_4]^-$ ion at *m/z* 97 [137].

19.4.2.3 Alkylethercarboxylates

The alkylethercarboxylates (AEC) ($C_nH_{2n+1}-O-(CH_2-CH_2-O)_n-COO^-$ H^+) belong to those compounds which were not yet found in environmental samples despite the fact that they are used more frequently for cleaning purposes in the households. Two different strategies applying API-FIA-MS-MS can be used for substance-specific identification of these compounds [136]. Applying CID in the positive mode leads to the loss of CO_2, as observed with the loss of SO_3 in alkylethersulfates applying MS-MS. Under these conditions the precursor parent ions to be submitted to CID will be the alkylethoxylate ions. Therefore all AEC parent ions from a commercial blend subjected to API-CID($+$) result in a characteristic pattern of daughter ions containing alkyl as well as polyether fragments (*m/z* 57, 71 and 113 or 89, 133 and 177) pretending alkylpolyglycolethers. However, in addition a dominating daughter ion with *m/z* 117 can be observed which is unspecific for surfactants of the polyglycol ether type [136]. However, APCI and ESI-FIA-MS-MS($-$) spectra generated with lower sensitivity but higher selectivity contain the very specific daughter ions with *m/z* 101 as shown in Fig. 19.52. The demand for selectivity of daughter or parent ions for the confirmation of the compounds in environmental samples using FIA could be fulfilled with this daughter ion. [136].

19.4.2.4 Alkylphenolethercarboxylates

For detection of the anionic di-nonylphenolethercarboxylates (di-NPEOC) (($C_9H_{19})_2-C_6H_3-O-(CH_2-CH_2-O)_m-CH_2-CO_2^-$) the parent ion scan of *m/z* 345 in the negative ESI and APCI-FIA-MS-MS mode is very specific, as Fig. 19.53 proves. The daughter ion of *m/z* 345 is the most abundant ion under CID conditions besides the precursor parent ion, and it is the product of the loss of the carboxylated polyether chain resulting in the $[(C_9H_{19})_2-C_6H_3-O]^-$ ion [136].

The daughter ion spectra generated from the di-NPEOC ionized in the positive mode suffer from the destructive ionization, as reported in Section 19.3.2.6 and also observed in the ionization process of alkylethersulfates. Since positive ionization leads to the bond cleavage of the carboxymethylene moiety ($-CH_2-CO_2^-$), daughter ions generated under these conditions now are only characteristic for di-alkylphenolethoxylates (di-NPEO). In contrast to the negative ionization resulting in the intact di-NPEOC compound ions prior to MS-MS, in the positive FIA-MS mode at first ($-CH_2-CO_2^-$) is abstracted from the alkyl
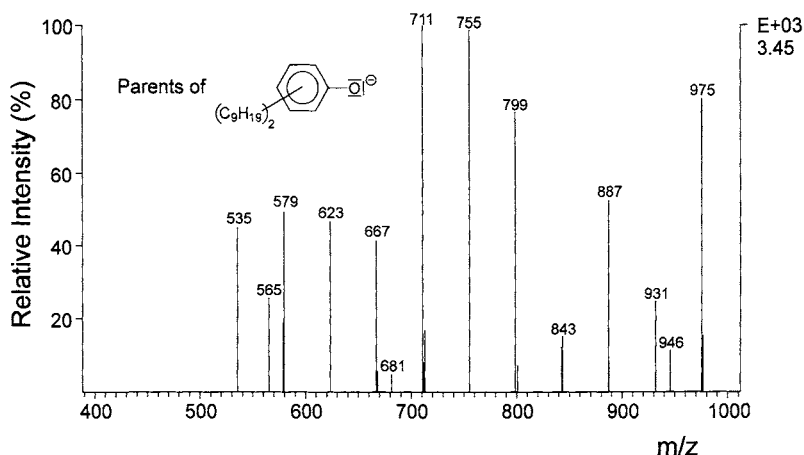
Fig. 19.53. APCI-FIA-MS-MS(−) (CID) parent ion mass spectrum of product ion with *m/z* 345 of di-nonylphenolethercarboxylate blend [136].

chain before both alkyl moieties are eliminated successively under positive FIA-MS-MS conditions ($[(C_9H_{19})_2-C_6H_3-O-(CH_2-CH_2-O)_m]^+$ → $[C_9H_{19}-C_6H_4-O-(CH_2-CH_2-O)_m]^+$ → $[C_6H_5-O-(CH_2-CH_2-O)_m]^+$) from the ions. Besides these daughter ions alkyl fragments (*m/z* 71, 85), polyether chain fragments (*m/z* 89, 133) and fragments as shown in Fig. 19.12 (Section 19.3.1) with different polyether chain length could be observed (*m/z* 121, 165) [136].

19.4.2.5 Fluorinated compounds

The mixture of anionic surfactants (cf. Section 19.3.2.7) consisting of alkylphosphinic and -phosphonic acids with perfluorinated alkyl moieties was examined by ESI-FIA-MS-MS in the negative mode [136]. The $[M − 1]^-$ ions of at *m/z* 399, 499 and 599 originating from the phosphonic acids ($C_nF_{2n+1}-P(O)(OH)_2$) resulted in a single negative daughter ion at *m/z* 79 with high intensity ($[PO_3]^-$), as observed under TSP(−) ionization, too [59,126].

The second main component of the mixture is phosphinic acid ($C_nF_{2n+1}(C_mF_{2m+1})-P(O)OH$ ($n = l \neq m$) containing perfluoro alkyl chains [59]. Applying CID to the negative ion at 801 ($n + m = 14$), according to the collision energy applied fragment ions with *m/z* 401 (60 eV) or 401 and 501 (80 eV), respectively, arise. These daughter ions were generated by MS-MS resulting in an abstraction of one of the perfluorinated alkyl chains. These chains either contain six or eight CF_2 links representing a 300 or 400 u mass loss from the parent ion with *m/z* 801 [136].

19.4.3 Cationics

19.4.3.1 Quaternary ammonium compounds

One of the first results dealing with MS-MS investigations of quaternary ammonium compounds (quats) was published by Conboy et al. [137]. Industrially important quats were separated by ion chromatography and determined by ion spray mass spectrometry in

the ESI(+) mode. Full scan IC-MS-MS spectra were presented from tetraalkylammonium compounds examined. They varied in the alkyl groups, starting at C_3H_7 (propyl) and ending at C_6H_{13} (hexyl).

API combined with an ion trap operating in ESI-FIA-MS(+) mode was found to be useful in meeting analysis needs for quats applied in products for personal hygiene (cf. Section 19.3.3.1). Blends of these quaternary ammonium surfactants with the general formula $(R)_nN^{\oplus}(CH_3)_{4-n}$, where R can be alkyl chains of 12–22 or more carbon atoms, were studied by ESI-FIA-MS-MS(+) for identification. These compounds were analyzed according to their chain length distribution [76]. MS-MS and MS^3 by CID almost exclusively resulted in sequential losses of intact long alkyl chains. A H̊ transfer from alkyl chain to the nitrogen resulted in an alkene. The behavior under CID conditions using ion trap mass spec is described as similar to that observed on tandem quadrupoles [76].

A mixture of quats of the general formula $RR'N^{\oplus}(CH_3)_2X^-$ could be classed as alkyldimethylbenzylammoniumacetate. The compounds contained $R = C_nH_{2n+1}-$ ($n = 12$, 14 and 16), $R' =$ benzyl ($C_6H_5–CH_2–$) and $X^- =$ acetate. Under ESI-FIA-MS(+) $[M]^+$ ions were exhibited at m/z 304, 332 and 360. The ion with m/z 304 of this commercial blend was analyzed by ESI-FIA-MS-MS(+), giving rise to the fragments with m/z 212 and 91 [67]. These daughter ions at m/z 212 or 91 proved the elimination of 92 u (loss of the neutral part toluene generated from the benzyl moiety after H transfer) resulting in m/z 212 and the generation of a benzyl cation $[C_6H_5–CH_2]^-$ with m/z 91 [67].

The commercially available surfactant $C_{13}H_{27}C(O)N(H)CH_2CH_2N(CH_2CH_2OH)$ $CH_2CH(OH)CH_2N^{\oplus}RR'R''$ ($R = R' = R'' = CH_3$; m/z 430) containing a quaternary center was examined by ESI-FIA-MS^n in the positive mode using ion trap (IT) [78]. The purpose was to establish a daughter ion library from which parent ions could be identified by reverse interpretation of daughter ion data. A map of ions generated in the different steps under MS^n ($n = 1–5$) was presented. Fragmentation behavior showing the H̊ transfer from the alkyl chain to the nitrogen and the loss of trimethylamine was proposed. The results of MS^n experiments and the 'explosion of information' available by these experiments were discussed. Differences in the fragmentation behavior of the ions applying TSQ or IT were mentioned [78].

Esterquats applied in the household as textile softener $(R(CO)OCH_2CH_2)_2–$ $N^{\oplus}(CH_3)CH_2CH_2OH \ X^-$; R = tallowyl or oleyl moieties; quaternary carboxyalkyl ammonium compounds) in the form of industrial blends were examined using APCI- and ESI-FIA-MS-MS(+). On the one hand interpretation of the APCI-MS-MS results was not possible because rearrangement reactions in the gas phase led to very complex spectra with large numbers of daughter ions. On the other hand ESI-FIA-MS-MS(+) of the fragment ion of the dioleyl compound at m/z 428 (cf. Section 19.3.3.2) resulted in a spectrum containing only one fragment ion with m/z 309 and the composition $[C_{17}H_{33}(CO)OCH_2CH_2]^+$ [142].

Surfactants were determined in surface water and foam samples resulting from an overflow drop of the Saale river, a tributary of the Elbe river [81]. APCI-FIA- as well as -LC-MS(+/−) was applied using ammonium acetate for ionization support. Besides non-ionic surfactants the cationic surfactants of fatty acid polyglycol amine type with the general formula $(R-N^{\oplus}H((CH_2–CH_2–OH)_x)–(CH_2–CH_2–OH)_yX^-)$ were detected by APCI-FIA-MS. The pattern of several series of equally spaced signals with $\Delta m/z$ 44 (Fig. 19.3a–c; cf. Section 19.3.1) presents the polyglycol amines (★) appearing as [M +
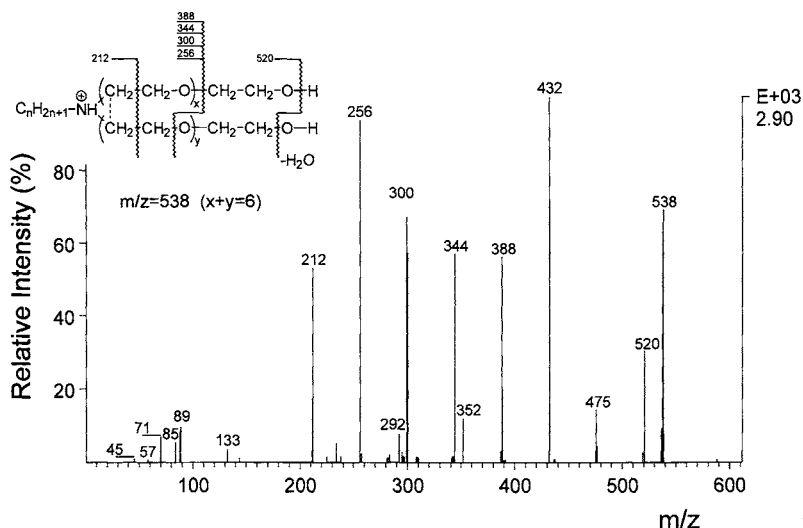
Fig. 19.54. APCI-FIA-MS-MS(+) (CID) daughter ion mass spectrum and fragmentation behavior of alkylpolyglycol amines homologue with m/z 538 from industrial blend [59].

H]$^+$ ions, as the daughter ion spectra proved. Separation of all mixture constituents by RP-C_{18} column chromatography was impossible, therefore the alkyl polyglycol amines could not be examined in the LC-MS-MS(+) mode. The polyglycol amines contained in the mixtures have to be eluted with an organic solvent mix used for column cleaning purposes [59]. The CID spectrum of the parent ion m/z 538 generated by FIA-MS-MS(+) resulted in a series of equally spaced daughter ions ($\Delta m/z$ 44) starting with 212 and ending at 520. Besides these daughter ions alkyl- and polyether fragment ions with low intensity were observed at 57, 71 and 85 or 45 and 89, respectively. The daughter ion spectrum of m/z 538 together with the fragmentation scheme is presented in Fig. 19.54 [67]. The results were confirmed by CID examination of two industrial blends characterized as 'alkylamine oxalkylates' by their data sheets. Vice versa parent ion generation in the FIA-MS-MS(+) mode, e.g. of the ions with $\Delta m/z$ 44 starting at m/z 212 allows the confirmation of the polyglycol amines in the foam and river water samples.

APCI-FIA-MS-MS(+) in the parent mode was used for the confirmation of polyglycol amines in the Saale river, because LC-MS in the RP-C_{18} mode failed. Cationics were not eluted from the column even with an acetonitrile share part of 95% in the mobile phase. The parents of m/z 212 resulting in equally spaced ions at 274 up to 714 ($\Delta m/z$ 44) were characteristic for the Leomin C 80 compound [88].

A fluorine-containing cationic surfactant of quat type was examined by FIA-MS using APCI and ESI in the positive and negative mode (cf. Section 19.3.3.1). The compound (C_nF_{2n+1}–SO_2–NH–CH_2–CH_2–CH_2–$N^{\oplus}(CH_3)_3$ X^-) was the first cationic surfactant that could be ionized in the negative mode, although it contained ammonium nitrogen. ESI-FIA-MS(+) mainly produced the [M]$^+$ ions at m/z 599 besides a small part of dealkylated ions ([M $-$ CH_2]$^+$) at m/z 585 [67]. The [M]$^+$ ions at m/z 599 were submitted to ESI-FIA-MS-MS(+) resulting in fragments at m/z 60 ([$(CH_3)_3NH]^+$), 72, 88 and 116 ([M $-$ (NH–CH_2–CH_2–CH_2–$N(CH_3)_3$)]$^+$. The dealkylation step [141] of the [M]$^+$ ion resulting in an

m/z: 342   m/z: 240
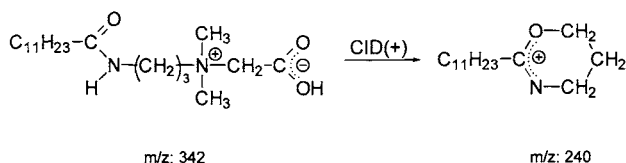
Fig. 19.55. Fragmentation behavior of C_{11}-betaine blend under CID conditions applying ESI-FIA-MS-MS(+) [142].

ion at 585 led to parallel daughter ions ($\Delta m/z$ 14) at 46 and 58. In addition a dominating fragment ion at m/z 85 appeared under these conditions [67].

19.4.4 Amphoterics

19.4.4.1 Betaines

The industrial blend of a betaine was examined by ESI-FIA-MS-MS(+) analysis (cf. Section 19.3.4.2). The compound was characterized by the general formula C_nH_{2n+1}–C(O)–NH–(CH$_2$)$_3$–N$^{\oplus}$((CH$_3$)$_2$)–CH$_2$–COO$^-$ H$^+$ resulting in a molar mass of 342. The [M + H]$^+$ ions with m/z 343 in the positive mode can be observed for the compound with $n = 11$. Applying CID the abstraction of 103 u (N((CH$_3$)$_2$)–CH$_2$–COOH) was observed resulting in the single dominating fragment of m/z 240 ([$C_{11}H_{23}$–C(O)–NH–(CH$_2$)$_3$]$^+$) thermodynamically favoured because of the ability to build up the cyclic structure shown in Fig. 19.55 [142].

Electrospray MS^n analysis leading to sequential product ions was used for the identification of an unknown surfactant in an extract of a shampoo formulation [77]. The compound could be identified as a betaine surfactant. An unusual series of daughter ions was detected at the MS^3 stage. The transition of the product ions at m/z 95 and 109 to ions at 67, 81 and 67 observed in MS^4 experiments together with other spectral observations led to the hypothesis that the unknown compound was a N-(2-aminoethyl) fatty amide with the general formula R–C(O)–NH(CH$_2$–CH$_2$–N)R'R". This could be confirmed by an authentic sample of the proposed laury amphomono acetate (LAMA) with the [M + H]$^+$ ion at 345 and R' = –CH$_2$–CH$_2$–OH and R" = –CH$_2$–CH$_2$–COOH. The fragmentation observed led to intensive examinations of amides. Only two of them, lauryl diethanol amide ([M + H]$^+$: 288), a non-ionic surfactant [18] and laurylamido-β-propyl betaine ([M + H]$^+$: 343), showed a similar behavior as LAMA. MS-MS examination of surfactants on a TSQ mass spec led to the explanation that a gas-phase rearrangement prior to the fragmentation had happened or an isomer had been present. MS^3 experiments by source CID and MS-MS in the second quad of TSQ produced ions consistent with the ESI ion trap [77].

The authors presented the whole information about the fragmentation behavior of a surfactant of coco amphomonoacetate type (R–C(O)–NHCH$_2$–CH$_2$–N(CH$_2$COOH)(CH$_2$–CH$_2$–OH)) from a shampoo mixture in a more detailed way, applying both positive ([M + H]$^+$; m/z 345) and negative ([M − H]$^-$; m/z 343) ionization in combination with MS^n [154]. In this way the complementary information of negative daughter ion examination allowed unequivocal identification. The positive as well as negative fragmentation scheme with dominant daughter ions and data display summarizing the negative ion MS^n behavior

of *m/z* 343 of this compound under electrospray ionization conditions was presented. Results were obtained either by an ion trap or a triple quad tandem mass spec, and it was concluded that MS^n provided greater insight than MS-MS alone [154].

19.4.5 Metabolites

19.4.5.1 Metabolites of non-ionics

Publications on the MS-MS identification of metabolites originating from surfactants are very rare in literature, however, the number of contributions dealing with substance-characteristic identification by MS methods increases. Especially for this type of compounds MS methods are more advantageous than non-substance-specific detection and identification techniques. Small or large structural modifications may occur disguising the precursor structure in the biogenic compound after biochemical degradation. This may influence the separation properties of the reaction products dramatically.

The most prominent metabolites of surfactants are polyethers of the ethylene and propylene type used for synthesis of non-ionics, anionics and even cationics. These compounds, which not only result from biochemical degradation processes but are also of anthropogenic origin, were degraded to carboxylic and dicarboxylic metabolites [96]. In addition carbonylic metabolites of PPG, which remind of the development of metabolites with analogous structure in the polyether chain, could be detected and identified for the first time by TSP using LC-MS and FIA-MS-MS, too [18,103]. Ions of polyether chains containing mixtures of ethylene and propylene glycol links originating from biodegraded EO/PO block polymers (cf. Section 19.3.1.2, Fig. 19.9) were not yet found and confirmed.

PEG as metabolites from non-ionic surfactants of alkylethoxylate type were detected in waste water, surface water, seawater and groundwater samples. Besides these polyglycolethers mono-carboxylated PEGs (MCPEG) and di-carboxylated PEGs (DCPEG) were found in form of their methyl esters using ESI-LC-MS(+). The full-scan total ion mass traces were presented in Fig. 19.38, however, no MS-MS results were presented [86].

Besides polyethylene glycols (PEG) their biochemical oxidation products were present in the tannery waste waters. Source CID(+) applying LC-MS in the APCI(+) mode resulted in fragmentation exhibiting characteristic ions of PEG as well as their mono and di-carboxylated biointermediates [99].

Surfactants of different types were found in a WWTP effluent discharged into river Saale, a tributary of the Elbe river [81,88]. Surface water samples and foam resulting from an overflow drop were examined by FIA-APCI(+) analysis. In addition it was assumed that besides non-ionic surfactants of the alkylethoxylate type metabolites of these compounds were present because of a series of equidistant ions (Δ *m/z* 44) parallel (Δ − 2 u) to other series of ions. This pattern of signals is characteristic for biochemical degradation products of non-ionics and polyethylene or polypropylene glycol ethers [59]. It can also be found in the degradation of polypropylene glycol [79]. To confirm the presence of carbonylic metabolites in the water samples, MS-MS in the FIA-APCI(+) mode by generating the parent ion spectrum of *m/z* 87 was applied. A series of homologue compounds, metabolites of non-ionic surfactants of alkylpolyglycol ether type (C_nH_{2n+1}–$O(CH_2CH_2O)_{m-1}$–CH_2CHO) in low concentration and predominantly carbonylic PEG metabolites ($HO(CH_2CH_2O)_x$–CH_2CHO) could be confirmed by this parent ion scan

[81]. Using the parent scan of 115, carbonylic metabolites of polypropylene glycol [79] and polypropylene glycol ethers [103] can be recognized.

In water samples of the Saale river, which contained complex mixtures of anionic and cationic surfactants, coelution effects were observed in the SPE concentration and elution procedure. Furthermore APCI-FIA-MS(+) did not allow representative overview spectra because of discrimination effects in ionization resulting from cationics. However, confirmation of PEG as metabolites of polyether-surfactants was successful by APCI-FIA-MS-MS(+). The positive parent ion scan of 89 was applied resulting in prominent ions which were not discriminated by cationics present in the mixtures. The ions were characteristic as PEG (m/z 256 up to 564, Δ m/z 44) besides traces of nonionics appearing, too [88].

Screening analysis for non-ionic surfactants of alkyl and arylethoxylate type in influent and effluent extracts of the Thessaloniki WWTP, Greece, applying APCI or ESI-FIA-MS(+) had for result that polyethylene and polypropylene glycol as metabolites of non-ionic surfactants were detected and identified by MS-MS [79].

So FIA-CID(+) using APCI and ESI confirmed that the parent ion at m/z 476 belonged to a PEG homologue because of its fragments at m/z 45, 89, 133, 175 and 221 originating from the polyether chain containing 10 (CH_2–CH_2–O) units [79].

In addition the effluent of the same WWTP contained two parent ions with m/z 266 and 324. FIA-MS-MS(+) spectra confirmed that both ions were homologues because of their identical fragmentation pattern [79]. Compounds could be characterized as secondary metabolites of the biochemical degradation product of polypropylene. Applying the diagnostic parent ion scan m/z 115 [81] to the mixture containing these compounds, ions at m/z 266 and 324 can be observed [79]. The PPG metabolites now contain one hydroxyl and one carbonyl function in the molecules (HO-[$CH_2(CH_3)$–CH_2–O-]$_n$–$CH_2(CH_3)$–CHO). This degradation pathway was described for alkylpolypropyleneglycolethers but not yet for PPG [18,103].

The persistant biochemical degradation products of nonylphenol ethoxylate surfactants were determined and characterized by ESI-MS(+) after LC separation [146]. Nonylphenolethercarboxylates, NPEC (C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)$_{m-1}$–CH_2–COOH), as well as alkyl chain carboxylated NPEOs, CNPEO (HOOC-(C_nH_{2n})$_x$–C_6H_4–O–(CH_2–CH_2–O)$_m$–H) and compounds carboxylated in both positions, CNPEC (HOOC-(C_nH_{2n})$_x$–C_6H_4–O–(CH_2–CH_2–O)$_{m-1}$–CH_2–COOH) were identified by the application of so-called source CID. For identification purposes the compounds were derivatized by methylation. The fragmentation behavior of the methyl esters of the acidic compounds as well as the underivatized compounds are presented in Fig. 19.56. The alkyl chain branched CNPE$_1$C compounds were confirmed as extremely recalcitrant intermediates in the biochemical degradation process [146].

The short-chain NPE$_0$Cs (C_9H_{19}–C_6H_4–O–CH_2–COOH), synthesized as standards, were examined applying APCI-FIA-MS-MS in the positive and negative mode. CID(+) generated the prominent [C_9H_{19}]$^+$ daughter ion besides alkyl fragments with low intensity at m/z 71 and 85. The negative ions developed only one negative ion by loss of CO_2 from the carboxylated chain resulting in the ion [C_9H_{19}–C_6H_4–O]$^-$ at m/z 219 [72].

The acidic metabolites (C_nF_{2n+1}–(CH_2–CH_2–O)$_m$–CH_2–COOH) besides short PEG chain metabolites were generated by biochemical degradation of the non-ionic fluorine-containing surfactant (C_nF_{2n+1}–(CH_2–CH_2–O)$_x$–H) in a lab-scale waste water treatment
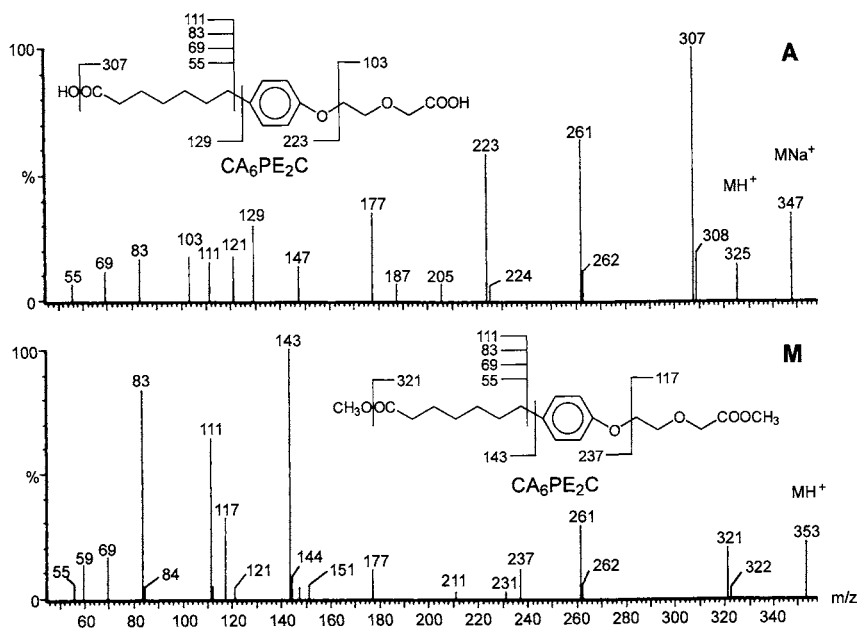
Fig. 19.56. Fragmentation behavior of the acidic NPEO metabolites (CNPEC); (A) underivatized compound and (M) di-methyl ester of $HOOC(CH_2)_6-C_6H_4-O(CH_2CH_2O)COOH$ under source CID conditions applying ESI-LC-MS(+) [146].

process (cf. Section 19.3.5.1) [17,59,92]. In contrast to TSP(+) ionization, ESI-FIA-MS(+) produced a series of equally spaced ions starting at m/z 484 ($n = 6$; $m = 2$) up to 834 ($n = 6$; $m = 10$). ESI-FIA-MS-MS(+) was used for confirmation of these metabolites generated with the same pattern as found by TSP CID [59]. Besides these metabolites the short-chain precursor fluorine-containing surfactants ($C_nF_{2n+1}-(CH_2-CH_2-O)_x-$H) were present; however, the Gaussian partitition originating from synthesis process was lost. The dominating homologue of the precursors contained 3 PEG units ($x = 3$). These compounds, now metabolites, can be regarded as final degradation product of successive PEG cleavage from long chain homologues, persistent against further degradation. ESI-FIA-MS-MS(+) applied confirmed these findings, because the TSP daughter spectra [17,59,92] were identical with ESI- results [104].

19.4.5.2 Metabolites of anionics

Metabolites of LAS found in interstitial water and seawater samples at several sampling points in a salt marsh of the Bay of Cadiz were characterized by ESI-LC-MS-MS(−) applying source CID [53]. The results proved the assumption that these metabolites were sulfophenylcarboxylic acid derivatives (SPC; $CH_3-(CH_2)_n-CH(C_6H_4SO_3H)-(CH_2)_x-$COOH), which varied by their alkyl chain lengths in the molecules. Daughter ion spectra were generated after RP-C_{18} separation in the source-CID(−) mode resulting in the spectra presented in Fig. 19.57. Despite the fact that all compounds characterized differ because of their alkyl chain lengths all metabolite homologues show the daughter ion at m/z 183,
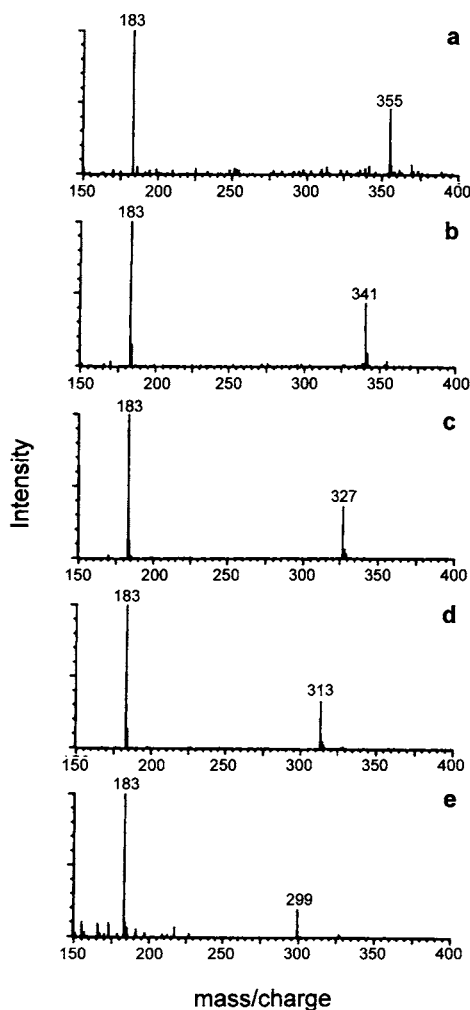
Fig. 19.57. Full-scan ESI-LC-MS(−) mass spectra of the (a) C_{12}, (b) C_{11}, (c) C_{10}, (d) C_9 and (e) C_8 SPC compounds of LAS SPE from water samples [53]. © 1997 by American Chemical Society.

characteristic for LAS. The source CID(−) examination covered the C_8 to C_{12} homologues with ions at m/z 299, 313, 327, 341 and 355 ($n + x = 5$–9). A connection between the adjustable ionization conditions (cone voltages) and the abundance of daughter and precursor parent ions in the CID spectra could be recognized [53].

Monitoring the surfactant load of WWTP influent and effluent (city of Thessaloniki, Greece) by APCI and ESI applying FIA- and LC-MS and -MS-MS in the negative mode the [M − H]⁻parent ions at m/z 297, 311, 325, 339 as well as 295, 309, 323, 337 and at m/z 299, 313, 327, 341 were detected in the effluent. The CID spectra in the FIA mode and in addition LC-MS proved that all these compounds should originate from LAS, but were modified by biochemical degradation. This assumption was confirmed because the LC behavior of these compounds was different from LAS. This was not only observed for
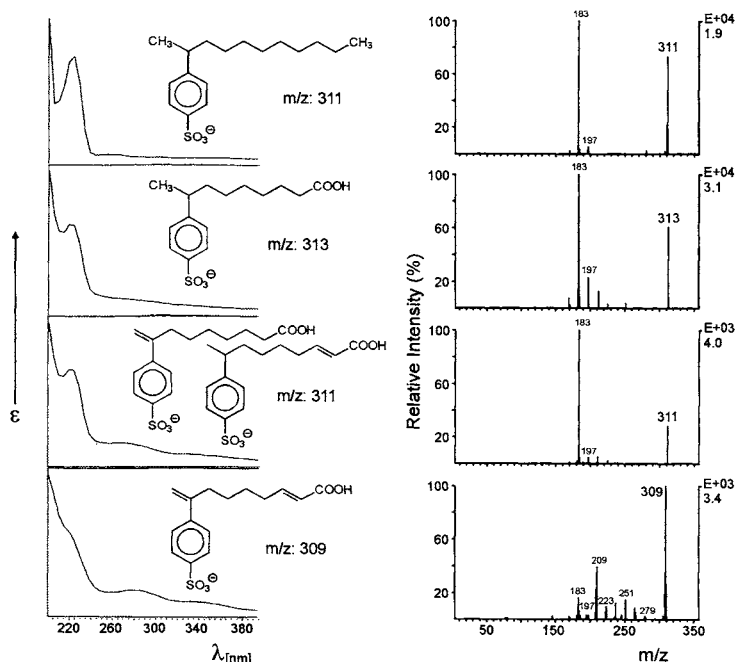
Fig. 19.58. Fragmentation behavior of LAS metabolites from C_{18}-SPE of WWTP applying ESI-FIA-MS-MS(−), their UV spectra and proposed structures [135].

compounds varying by $\Delta m/z \pm 2$ from the LAS ions at m/z 297, 311, 325, 339 but also for these compounds as isomers of LAS [135]. The characterization and identification of the compounds contained in the effluent was not possible using only MS-MS data. UV spectra and MS-MS spectra recorded in parallel allow the conclusion that all compounds ionized by FIA-MS(−) in the methanol fraction of the effluent were metabolites of LAS as shown in Fig. 19.58. The compounds with ions at m/z 299, 313, 327 and 341 detected here seemed to be identical with compounds found in the Bay of Cadiz [53], i.e. long-chain carboxylic metabolites of LAS (SPCs $(CH_3–(CH_2)_n–CH(C_6H_4SO_3H)–(CH_2)_x–COOH)$. The other compounds with ions at m/z 295, 309, 323, 337 or 297, 311, 325, 339, respectively, are classed with LAS metabolites with unsaturated alkyl chain because of their UV absorbances [135].

Short-chain intermediates of LAS carboxylated in the alkyl chain (SPC) (Fig. 19.40 I–III) and the calcium salts of CPC (Fig. 19.40 IV) had been synthesized as standard compounds for the identification of potential LAS metabolites [54,147]. ESI-LC-MS(−) studies proved that these compounds were extremely polar, because they show no retardation in RP-C_{18} chromatography [72]. ESI-FIA-MS-MS(−) of all calcium salts resulted in two daughter ions originating from [M − 1]$^−$ ions at m/z 215, 229, 243 and 257 with the structural formula presented in Fig. 19.40 I–IV. The first daughter ion at m/z 80 is [SO_3]$^−$ and the second ion at m/z 170 belong to the resonance hybride of the [$H_2C–C_6H_4–SO_3$]$^−$ ion. These two characteristic daughter ions can be used for the recognition of these compounds in complex mixtures applying in the ESI-FIA-MS-MS(−) parent ion scans of
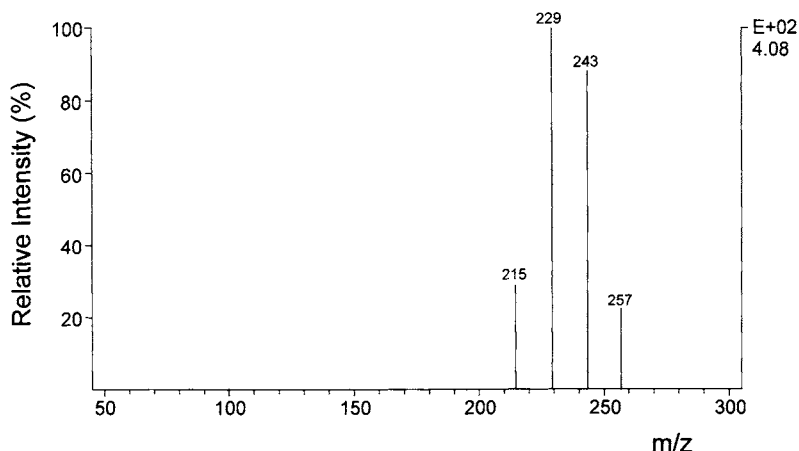
Fig. 19.59. ESI-FIA-MS-MS(−) parent ion mass spectrum (PIS) of m/z 80 as diagnostic scan for short-chain carboxylates of LAS [72].

80 or 170 as presented in Fig. 19.59 [72]. ESI-FIA-MS-MS(−) of the SPCs, however, resulted in daughter ion spectra showing ions at m/z 80 ($[SO_3]^-$) and 183 ($[CH_2=CH-(C_6H_4)-SO_3]^-$) as observed in CID(−) spectra of LAS, too [72].

19.4.5.3 Biogenic surfactants

The rhamanolipids with the general structural formula as shown in Fig. 19.43 (Section 19.3.5.6) can be ionized with three dominating ions at m/z 376, 522 and 668 using ESI-FIA-MS(+). All daughter ion spectra received from these homologues by ESI-FIA-MS-MS(+) contained the four prominent ions at m/z 153, 171, 189 and 359 besides few other ions with low intensity. These ions were not yet characterized by their structure [80].

In contrary to these results the cyclic sorphose lipids shown with its general structural formula in Fig. 19.44 contained a lot of daughter ions after ESI-FIA-MS-MS(+). However, the daughter ions from these three parent ions at m/z 704, 706 and 708, varying because of one, two or three double bonds in the molecule, present a characteristic pattern of ions. The only variation by $\Delta m/z$ 2 in these series of daughter ions was observed according to the variations in the unsaturated alkyl chain of the molecules [80].

19.5   QUANTIFICATION OF SURFACTANTS AND THEIR METABOLITES BY FLOW INJECTION ANALYSIS (FIA) AND AFTER LC-SEPARATION IN COMBINATION WITH MS-DETECTION

For a long time exclusively and even up to now quantification of surfactants in waters was done by substance-class-specific determination methods combined with all interferences to compounds with similar structure or discrimination reactions. Therefore false positive or negative results may happen [28,29]. As toxicity testing exhibited quite different results according to the molecular structures of the same surfactant type and even to the

different homologues from the same surfactant [2,3] great efforts were made during the last years to develop substance-specific surfactant determination methods for all kind of matrices like industrial blends, drinking water, surface water and waste water samples [31,59,155]. Otherwise it was impossible to make precise statements on the effects of these compounds on the aquatic organisms in the waste water treatment process as well as in the rivers used as receiving waters. For this reason MS detection after LC separation or using FIA has become one of the most applied effective methods. In the first time of on-line-coupled MS detection thermospray ionization was the method of choice for the substance specific quantification of surfactants and their metabolites [59], whereas today API-methods like APCI or ESI are preferred. MS detection in the quantification process presents the great advantage that mass-selective detection, which sometimes is synonymous with substance-specific recognition, is provided. This is helpful and often essential if high concentrations of matrix compounds cannot be separated by chromatography and, thus, would disturb the non-substance-specific optical detection techniques. Mass-selective detection for quantification may be carried out after LC separation (LC-MS) or in the flow injection mode bypassing the analytical column (FIA-MS). If the sample throughput is decisive for the choice of the quantification method, FIA-MS is predominantly applied [15,59,66,156]. Under FIA conditions using only MS, however, problems may arise because of the presence of different compounds with identical m/z-ratio, resulting in false positive elevated concentrations.

But if compounds in complex mixtures have to be quantified in the FIA mode, i.e. applying mixture analysis because of insufficient separation, specificity can be improved by using MS-MS techniques. Daughter and parent ion scans as well as neutral loss are alternatives applicable as substance-specific methods in the FIA-MS-MS mode bypassing the analytical column [40,59,88,156]. But under these conditions discrimination reactions have to be expected because of the varying tendency of the mixture constituents to be ionized. To minimize ionization suppression by interfering compounds LC separation is the method of choice, and sometimes it is essential. The highest level in reliability can be reached in the quantitative determination of compounds if MS-MS is applied during LC separation (LC-MS-MS), but quantification under these conditions is very time-consuming.

A great obstacle to all quantitative determinations of unknown compounds, e.g. surfactants by MS or MS-MS is that the identity of the compound to be quantified must be known. But the greatest obstacle for quantification is that in addition the compound should be available as standard, too, because the response factors of the substances, which slightly differ in their molecular structure, may considerably vary. This response factor depends on the efficiency the compounds can be ionized under positive or negative conditions. If the identified compound is not available as standard, determination should be carried out using a compound with a comparable response, e.g. homologues or compounds with similar structure and/or elemental composition. Under these conditions quantification is possible on the assumption that the compounds used as surrogate quantification standard and the compound to be analyzed have comparable response factors. However, quantification results provided under these conditions have to be checked very critically. The non-availability of standards for surfactants and biochemical degradation products of all surfactants, which are not very common, is the reason that not much research work was done in the past. The number of papers dealing with this topic increased during the last

years, but only one comparable systematic approach to quantitative determination of primary alcohol ethoxylate surfactants, as presented by Evans et al. [157], was published up to now applying the new API methods [22]. Actual systematic quantifications of non-ionics and anionic surfactants in spiked real environmental samples (waste water) applying API methods in the LC- and FIA-MS mode were performed in an interlaboratory testing program [158].

19.5.1 Quantification by flow injection analysis (FIA) and after LC-separation

19.5.1.1 Non-ionics

Quantitative determination of alcohol ethoxylate surfactants (AEO; C_nH_{2n+1}–O–$(CH_2$–CH_2–O$)_m$–H; $n = 12$, 13, 14, 15, 16 and 18) and nonylphenolethoxylates (NPEO) in industrial blends, drinking water, river water, raw and treated waste water was carried out after GCB-SPE (graphitized carbon black) concentration and elution with dichloro-methane/methanol (80/20; v/v) using ESI-LC-MS(+) [22] after drying in a stream of nitrogen and dissolution of the residues in methanol/water (70/30; v/v). Micro-LC separation using a gradient elution starting with 80% methanol and 20% water containing 0.1 mmol/l of trifluoroacetic acid (TFA), programmed linearly to 100% of methanol after 20 min, was done on a Alltech C-8 reversed phase material. A C-10 EO_6 AEO was used as quantitative internal standard. Prior to quantification the linear dynamic range of the ESI mass spec was investigated comparing the ion signal intensity vs. the amount of C-11 AEO starting with 1 ng and going to 80 ng standard injected. A linear dynamic range was found for 1–20 ng of C-11 EOs injected onto the column. The effect of EO chain length on the ion signal intensity was assessed by measuring peak areas of AEO compounds with identical alkyl chain length but increasing EO number in full-scan mode. Determination of the ion signal intensity of AEO homologues with different alkyl chain length and 6 EO units in the polyether chain resulted in different response factors for these surfactants reaching from 1.0 for C-10 and C-12 to 1.07 for C-14, 1.08 for C-16 and 1.16 for C-18 [22]. In parallel between-day precision was assessed over a measuring period of 3 weeks.

The limits of detection (LOD) under these conditions were approximately 20 pg of each AEO homologue injected onto the column, resulting in LOD of 4 pg using ESI because of a split ratio of 4:1 favouring waste and a S/N ratio of 10.

Results of recovery experiments in drinking water, river water, raw and treated waste water as well as from real samples, originating from three mechanical/biological waste water treatment plants were reported. Elimination efficiency for AEOs was >95%, however, resulting in an increased amount of polyethylene glycol homologues as meta-bolites of AEO biodegradation. NPEO elimination efficiency of waste water treatment was lower than for AEOs, but >95%, too [22].

Standards of non-ionic surfactants of the polyether type (AEOs and NPEOs), PEG as metabolites of non-ionic surfactants and carboxylated metabolites of polyethylene glycols (see Section 19.5.1.5) were determined quantitatively using APCI-LC-MS(+). Recovery rates and calibration curves were determined after spiking and automated single and sequential SPE concentration on LiChrolut EN and C_{18} material prior to the examination of real environmental samples of tannery waste water [99]. The LOD values were determined and repeatability studies were performed in the SIM or full scan mode.

Elution from SPE materials was done selectively using eluents with different polarities.

For LC separation a gradient elution using 1 ml/min on a Hypersil Green ENV column was applied starting isocratically for 10 min with a mixture of 50% eluent A (water) and 50% eluent B (acetonitrile), both acidified with 0.5% of acetic acid. After this the concentration of B was increased linearly to 100% in 25 min. After 5 min isocratic the gradient was changed within 5 min to the initial conditions, allowing separation of each individual homologue and ethoxymer [99]. Concentrations of AEOs in tannery waste water were reported varying between 0.33 and 1.13 mg/l for the different homologues, whereas NPEO concentration reached 3.09 mg/l for $NPEO_9$.

Non-ionic surfactants of alkylethoxylate (C_nH_{2n+1}–$O(CH_2CH_2O)_mH$) and alkylpropoxylate type (C_nH_{2n+1}–$O(CH(CH_3)CH_2O)_mH$), fatty acid diethanolamides (C_nH_{2n+1}–$C(O)$–$N(CH_2$–CH_2–$OH)_2$) and nonylphenolethoxylates (NPEO) were quantitatively monitored by FIA-MS-MS(+) in the Elbe and Saale river in Germany during several examination periods in the years 1995–1996 applying APCI or ESI ionization. C_{18}-SPE prior to selective elution by diethylether or methanol was used for concentration [81]. Mixture analysis by FIA-MS-MS(+) was applied using the diagnostic parent scans m/z 89, 117, 106 and 291 for the detection and identification of AEOs, alkylpropoxylates, fatty acid diethanolamides and NPEOs, respectively [40,88]. In addition NPEOs were quantified in some of these samples by LC-MS-MS(+) (m/z 291) for confirmation purposes using a Sperisorb 5 ODS column in combination with a linear gradient of acetonitrile (A) and methanol/water (B). Starting with 10% A the gradient reached 90% A within 45 min. Anthropogenic surfactant concentrations found reached maxima of about 45 μg/l for alkylglycolethers, <5 μg/l for diethanolamides and about 10 μg/l for NPEOs in the Elbe river [40,88].

Concentrations below 0.5 μg/l for alkylglycolethers and NPEOs were found in the water of the Saale river, whereas 40 and 1190 μg/l could be confirmed as maximum concentrations in foam samples collected from the Saale river, respectively [40,88].

Screening NPEOs in waste water samples using precursor ions of m/z 121 and 133 and multiple reaction monitoring in the API-FIA-MS-MS(+) mode, semiquantitative estimations about NPEO elimination were possible. Under the STP conditions it was observed that the removal rates for the homologues differed in dependence on their PEG chain length. Calculations of the total NPEO concentration in STP influent and effluent revealed that 78% of NPEO, i.e. 37 mg/l, were removed from the waste water by biological treatment. This elimination, however, based on a preferential metabolization of the EO_5–EO_{16} homologues of NPEO, resulting in short chain NPEOs [107].

19.5.1.2 Anionics

A blend of alkylethersulfates (AES; C_nH_{2n+1}–O–$(CH_2$–CH_2–$O)_x$–SO_3H) containing also alkylsulfates (AS; C_nH_{2n+1}–O–SO_3H) as unreacted compound from alkylethersulfate synthesis was used to develop a method for quantitative monitoring of these compounds in environmental matrices. RP-C_2 SPE material was applied for concentration and methanol/2-propanol (80/20; v/v) was used for elution prior to separation and negative MS detection. Ion spray LC-MS(−) was used to determine quantitatively interference-free individual AES species. The method applied enabled a complete resolved separation according to alkyl and ethoxylate chain length. Under these conditions it was possible to validate this method using spike and recovery and measuring concentrations of AES species with 12–15 alkyl groups and O–8ethoxylate units in the molecules [134]. River

water and influent and effluent samples of waste water treatment plants were examined after spiking allowing a monitoring to ppb levels in the full-scan mode. SIM and multiple injections enabled the monitoring of 50-fold lower concentrations. In this way it was possible to measure 224 and 346 μg/l of C_{12-13} and C_{14-15} AES in WWTP influent, 60 and 28 in WWTP effluent or 2.5 and 7.8 in river water as environmental pollution, respectively. A mathematical regression model was presented providing prediction of total AES concentrations from measurements of selected homologues.

Separation was achieved with a linear gradient consisting of 0.3 mM ammonium acetate in acetonitrile/water (20/80; v/v) as mobile phase A and 0.3 mM ammonium acetate in acetonitrile/water (80/20; v/v) as mobile phase B. The gradient was programmed from 80% A to 45% A within 30 min [134].

Long-chain intermediates carboxylated in the alkyl chain and their precursor compounds, the LAS were quantified from seawater samples taken from sampling points in a salt marsh of the Bay of Cadiz (Spain). ESI-LC-MS(−) [53] applying RP-C_{18} chromatography under ion-pairing conditions using methyl triethylammonia acetate was performed. The compounds were quantified after sequential acidic extraction from the seawater and interstitial water samples using C_{18} hydrophobic material and SAX (strong anionic exchanger) and elution with acidic methanol. Examinations had for result that with the distance from urban effluent discharge point the LAS concentrations in the samples decreased very quickly from approximately 200 to <10 μg/l, whereas the metabolite concentrations reached a maximum at a distance of about 3.5 km from the discharge point [53]. For LC conditions used see Section 19.5.1.5.

The anionic surfactant mixture of LAS was quantitatively monitored by FIA-MS-MS(−) in combination with APCI or ESI interface in the Elbe and Saale river in Germany in the course of four examination periods during the years 1995 and 1996. C_{18}-SPE prior to selective elution by methanol was used for concentration [81]. The mixture analytical approach for substance characteristic quantification by FIA-MS-MS(−) was applied using the negative diagnostic parent scan of m/z 183 for the detection and identification of LAS. Concentrations found reached maxima of about 40 μg/l for LAS in the Elbe river and 2 μg/l in the water of Saale river or 9360 μg/l in foam samples collected from Saale river, respectively [40,88].

Linear alkylbenzene sulfonic acid compounds (LAS) were found in seawater samples of the North Sea in concentrations of 30 ng/l. Quantitation was performed using electrospray LC-MS-MS(−) in the parent ion scan mode (m/z 183) after C_{18} SPE and methanol elution [26]. For LC conditions see [40,88].

19.5.1.3 Cationics

The quantitative surfactant contents of a WWTP discharge, surface water and foam resulting from an overflow drop were determined in a series of surface water examinations of a tributary of the Elbe river [81]. APCI-FIA-MS(+) and MS-MS(+) were applied to quantify the cationic surfactants of fatty acid polyglycol amine type (R-N$^{\oplus}$H((CH$_2$–CH$_2$–OH)$_{x,y}$)$_2$ X^{-}). For calibration purposes an industrial blend was used as standard. For concentration RP-C_{18} SPE in combination with selective elution was applied [40,88]. After selective elution with methanol/water and methanol the polyglycol amine fractions were ionized. Polyglycol amines with short polyglycolether chains could be observed in

the methanol/water eluate, whereas the alkyl polyglycol amines with long ether chains, i.e. with elevated m/z ratios dominate the methanol fractions both recognizable by their characteristic ions equal-spaced with $\Delta m/z$ 44. Methanol and methanol/water fractions were mixed, and the intensities of the ion currents of a selected number of the equally spaced ions starting at m/z 318 and ending at 758 were determined in SIM mode and used for quantification. The concentrations of alkyl polyglycol amines found in WWTP discharge, surface water and foam were 6, 0.01 and 20 mg/l, respectively [67].

19.5.1.4 Amphoterics

No results have been published for the quantification of amphoteric surfactants using API-FIA- or -LC-MS methods.

19.5.1.5 Metabolites

The biochemical oxidation products of polyethylene glycols, mono- (MCPEG; H–$(OCH_2CH_2)_nO–CH_2–COOH$) and dicarboxylate polyethoxylate glycols (DCPEG; $HOOC–CH_2(OCH_2CH_2)_nO–CH_2–COOH$), as secondary metabolites of the primary metabolite PEG were determined quantitatively in tannery waste waters. After automated single and sequential SPE concentration on LiChrolut EN and C_{18} material APCI-LC-MS($+$) was applied for quantification [99]. For LC separation conditions see Section 19.5.1.1. Under these conditions the primary metabolite PEG as well as MCPEG and DCPEG of different chain length were found in maximum concentrations of 0.028, 0.19 and 0.25 mg/l, respectively.

The quantitative analysis of primary (PEG) and secondary metabolites (carboxylated PEG) of non-ionic surfactants of alkylethoxylate type in waste water, surface water, seawater and groundwater samples was carried out using ESI-LC-MS($+$). The biodegradation behavior of PEG and related intermediates over a period of a maximum of 10 days was monitored. PEG, mono-carboxylated PEGs (MCPEG) and di-carboxylated PEGs (DCPEG) extracted before by SPE using graphitized carbon black (GCB) were determined. For extraction neutral and acidic conditions were applied [86,143]. Commercially available MCPEG and DCPEG homologues were used to perform recovery studies allowing statements about the concentrations in the media analyzed [86]. For this purpose the metabolites were derivatized as methyl esters. Concentration graphs of PEG, MCPEG and DCPEG homologues in seawater related to the number of ethoxylate units in the molecules according to the increased distances from Tiber's estuary were presented. Maximum concentrations of PEG, MCPEG and DCPEG were correlatated to the lowest salinity, reaching 14, 0.2 and 0.8 µg/l, respectively. Sewage treatment plant influents and effluents, however, contained several times as much of these concentrations [86].

The possible different metabolites of nonylphenolethoxylates, the nonylphenolethercarboxylates, NPEC ($C_9H_{19}–C_6H_4–O–(CH_2–CH_2–O)_{m-1}–CH_2–COOH$), as well as alkyl chain carboxylated NPEOs, CNPEO ($HOOC-(C_nH_{2n})_x–C_6H_4–O–(CH_2–CH_2–O)_m–H$) and compounds carboxylated in both positions, CNPEC ($HOOC-(C_nH_{2n})_x–C_6H_4–O–(CH_2–CH_2–O)_{m-1}–CH_2–COOH$) were examined by ESI-MS($+$) [146] in waste water treatment plant effluents. After SPE concentration on Carbograph material and elution with dichloromethane/methanol (80/20; v/v) acidified with formic acid the acidic metabolites were

quantified as methyl esters by ESI-MS(+) after LC separation. Because of the lack of standards quantification was based on an assumption about a comparable response of the precursor compounds. Therefore relative intensities of the ions of the carboxylated compounds were reported. According to this assumption a total amount of 58 µg/l of CNPECs, i.e. compounds carboxylated in both positions and 0.68 µg/l of CNPEOs, i.e. alkyl chain carboxylated NPEOs were determined in a selected sample. The CNPEC concentration of 58 µg/l accounted for 63% of NPEO metabolites leaving the waste water treatment plant [146].

Long-chain intermediates carboxylated in the alkyl chain were known as metabolites of the anionic surfactant mixture LAS ($CH_3-(CH_2)_n-CH(C_6H_4SO_3H)-(CH_2)_x-COOH$; sulfo-phenylcarboxylic acid; SPC; $n + x = 7$–13)). These so-called SPC compounds (see Section 19.3.5.5) were found and studied quantitatively from sampling points in a salt marsh of the Bay of Cadiz (Spain). ESI-LC-MS(−) applying RP-C_{18} chromatography under ion-pairing conditions using methyl triethylammonia acetate was performed [53]. After sequential acidic extraction from the water and interstitial water samples using C_{18} hydrophobic material and SAX (strong anionic exchanger) elution was performed with acidic methanol before the compounds were quantified. Since no standard compounds were available for SPCs quantification, these compounds were determined as C_{10}-LAS equivalents. The metabolite concentrations reached with 120 µg C_{10}-LAS equivalents/l a maximum at a distance of about 3.5 km from the discharge point, whereas in parallel the LAS concentrations in the samples decreased from approximately 200 below 10 µg/l in a distance larger than 12 km from discharge point [53]. The relative distribution of SPCs with different chain length found at the different sampling stations plotted against residence time of LAS was presented.

Separation of the SPCs was achieved on a Hypersil C_{18} column with a gradient consisting of acetonitrile/water (80/20; v/v) as mobile phase A and water as mobile phase B. Both mobile phases contained 5 mM triethylamine and 5 mM acetic acid. Gradient was programmed from 5% A to 60% A within 25 min and then to 100% A within the next 25 min [53].

19.6 CONCLUSIONS

Looking back to the past, mass spectrometric examinations up to now have become more and more important for the analysis of surfactants and their metabolites [59,65,83,143,159]. The techniques FIA-MS, LC-MS or -MS-MS applied for detection, identification and quantification in combination with the thermospray (TSP), fast atomic bombardment (FAB), particle beam (PB), electrospray (ESI), atmospheric pressure chemical ionization (APCI) or matrix-assisted laser desorption time of flight-technique (MALDI-TOF) have proved to be the methods of choice for substance-specific analyses on this group of compounds produced in industrial-scale quantities. Surfactants as polar compounds of anthropogenic origin are contained in waste waters, surface waters and even drinking water; however, since these compounds are not easily detectable, their presence and their effects in the environment, resulting from their specific bipolar structure, had been neglected for a long time.

The most important reason for this handling was that surfactants are not volatile without

TABLE 19.1

Determination, identification and quantification of surfactants and their metabolites by modern mass spectrometric techniques[1]

Compound	Sample/ matrix	Isolation	Mode	Determination		Identification		Quantification	
				LC	FIA	LC	FIA	LC	FIA
Nonionics									
Alkyl polyethylene glycol ethers	Standard	–	+	[97]^a	[95]^c [96,98]^b [99]^a [101]^{bb} [102]^d	–	[96]^b [99]^a [153]	[99]^a	–
	Blends, drinking, river, waste water	GCB-SPE	+	[22]^b	–	–	–	[22]^b	–
	River, waste water	C$_{18}$-SPE	+	[40,81]^a	[81]^a [88]^{ab}	–	[88]^{ab} [81]^a [40]^a	–	[88]^{ab} [40]^{ab}
	Waste water	C$_{18}$- LiChrolut EN-SPE	+	[79]^{ab}	[79]^{ab}	–	[79]^{ab}	–	–
	Waste water	C$_{18}$, GCB, LiChro-lut EN, SAX-SPE	+	–	[75]^b [100]^b	–	–	–	–
Alkyl polyethylene glycol ethers (fluorinated) PPG-type	Standard	–	+	[69]^{ab}	[69]^{ab}	–	[69]^{ab}	–	–
Alkyl polypropylene glycol ethers	Standard	C$_{18}$-SPE	+	[69]^{ab}	[69]^{ab}	–	[104]^a	–	[40,88]^{ab}
	Waste water	C$_{18}$- LiChrolut EN-SPE	+	–	[79]^{ab}	–	[79]^{ab}	–	–

TABLE 19.1 *(continued)*

Compound	Sample/ matrix	Isolation	Mode	Determination		Identification		Quantification	
				LC	FIA	LC	FIA	LC	FIA
Alkyl polyglycol ethers PEG/ PPG-type (EO/PO)	Standard	C$_{18}$-SPE	+	[104][a]	[104][a]	–	–	–	–
Alkyl phenol ethoxylates	Standard	–	+	[104][a]	[95][c] [99][a]	–	[99][a] [153]	[99][a]	–
	Blend	C$_8$-SPE	+	[109][a]	[105][a] [109][a]	–	–	–	–
	Blend	–	+	[108][a]	[98][b] [108][a]	–	–	–	–
	River water	C$_{18}$-SPE	+	[40,88][a]	[88][ab] (PIS) [40,81][a]	[88][a]	[40,81,88][a]		[40,88][ab]
	Waste water	C$_{18}$-SPE	+	[40,88][a] [79][ab]	[40,88][a] [79][ab]	–	–	–	–
	Waste water	–	+	–	[107] (PIS)		[107] (PIS)	–	[107] (PIS)
	Waste water	XAD-16	+	–	[110][ab]		[110][a]	–	–
	Waste, river and drinking water	GCB	+	[22][b]	–	–	–	–	–
Alkyl cyclohexanol ethoxylate	Standard	–	+	[104][a]	[99][a]	–	–	–	–
Fatty acid polyglycol esters	Blends	–	+	[111][b]	[111][b]	–	[104][b] [153]	–	–

Fatty acid diethanol amides	Blends	C$_{18}$-SPE	+	[69][b]	[69][ab]	–	[77,104][b]	–	–
	River water	C$_{18}$-SPE	+	–	–	–	–	–	[40,88][ab]
Unsaturated fatty acid diethanol amides	Blend	–	+	–	[104][b]	–	[77,104][b]	–	–
Alkyl polyglycosides	Blends	C$_{18}$-SPE	+/–	[69][a]	[69][ab]	–	[69][a]	–	–
Alkyl glucamides	Blend	–	+/–	[69][b]	[69][ab]	–	[69][a]	–	–
Polyethoxylated sorbitan derivatives	Blend	–	+	–	[95][c]	–	–	–	–
Polyethoxylated decyne diols	Blend	–	+	–	[112,113][bcefg]	–	–	–	–
Anionics									
Alkylbenzene sulfonic acid	Standard	–	–	–	[102][d]	–	[72,131][b] [136][ab] [137]	–	–
	Standard	–	+/–	[109][a]	–	–	–	–	–
	River water	–	+/–	[109][a]	–	–	–	–	–
	River water	C$_{18}$-SPE	–	[40,81,88][b]	[40,88][b]	–	[88][b]	–	[40,88][ab]
	Sea water	C$_{18}$-, SAX-SPE	–	[53][b]	–	–	–	[53][b]	–
	Sea water	C$_{18}$-SPE	–	–	–	–	–	[26][b]	–
	Waste water	C$_{18}$-, LiChrolut EN-SPE	–	[135][ab]	[135][ab]	–	–	–	–
	Waste water	C$_{18}$, GCB, LiChro-lut EN, SAX-SPE	–	–	[75][b]	–	–	–	–

TABLE 19.1 *(continued)*

Compound	Sample/ matrix	Isolation	Mode	Determination		Identification		Quantification	
				LC	FIA	LC	FIA	LC	FIA
	Waste water	—	—	—	—	—	[96][b]	—	—
Substituted benzene and naphthalene sulfonates	—	—	—	—	—	—	[131][b]	—	—
Alkane sulfonates	Blends	—	—	[136][a] [137][b]	[136][ab]	—	[81,136][ab]	—	—
	Waste water	C[18], GCB, LiChrolut EN, SAX-SPE	—	—	[75][a]	—	—	—	—
Alkenesulfonates	Blend	—	—	[136][a]	—	—	—	—	—
Alkylsulfates	Blends	—	—	[136][a] [137][b]	[102][d]	[137][b]	[136][a]	—	—
	Waste and river water	C[2]-SPE	—	[134][b]	—	—	—	—	—
Alkyl ether sulfates	Blend	—	—	—	[102][d]	—	—	—	—
	Blend	C[18]-SPE	+/−	[70][ab]	[70][ab]	—	[70][ab]	—	—
	Formulat.	—	+/−	—	[96][b]	—	—	—	—
	Waste and river water	C[2]-SPE	—	[134][b]	—	—	—	[134][b]	—
Alkyl ether sulfates, EO/PO-mixture	Research sample	—	+/−	—	[132][ab]	—	—	—	—

Alkyl ether carboxylates	Blend	C_{18}-SPE	+/−	[136][a]	[136][ab]	−	[136][ab]	−	−
Alkyl aryl ether sulfonates	Blend	−	+/−	−	[136][ab]	−	−	−	−
Alkyl aryl ether sulfates	Blend	−	+/−	−	[136][ab]	−	−	−	−
Alkyl aryl ether phosphates	Blend	−	+/−	−	[136][ab]	−	−	−	−
Dialkyl aryl ether carboxylates	Blend	−	+/−	[136][a]	[136][ab]	−	[136][ab] (PIS)	−	−
Phosphonic/ phosphinic acid (fluorinated)	Blend	C_{18}-SPE	−	[136][ab]	[136][ab]	−	[136][b]	−	−
Sulfosuccinates	Blend	−	+/−	−	[136][a]	−	−	−	−
	Blend	−	−	−	[102][d]	−	−	−	−
Alkyl polyglucoside esters	Blend	−	+/−	−	[140][b]	−	−	−	−
Cationics Quaternary alkyl ammonium compounds	Blends	−	+	[137][b]	[67][ab] [34,76][b]	[137][b]	[76,78][b] [67][ab]	−	−

TABLE 19.1 *(continued)*

Compound	Sample/ matrix	Isolation	Mode	Determination		Identification		Quantification	
				LC	FIA	LC	FIA	LC	FIA
Quaternary carboxyalkyl ammonium compounds (esterquats)	Blends			–	[142][ab]	–	[142][ab]	–	–
Alkoxylated ammonium salts (fluorinated)	Blend	–	+/–	–	[67][ab]	–	[67][ab]	–	–
Fatty acid polyglycol amines	Waste and river water	C$_{18}$-SPE	+	[88][a]	[40,88][a]	–	[67,88][a]	–	[67][a] [40,88][ab]
Amphoterics									
Amine oxides	Blends	–	+	–	[142][ab]	–	–	–	–
Betain	Blends	–	+	–	[96][b]	–	[77][b]	–	–
Betain	Blends	–	+/–	[142][b]	[142][ab]	–	[142,156][b]	–	–
Betain (sultaine)	Blend	–	+/–	[142][b]	[142][ab]	–	–	–	–
Metabolites									
Polyethyleneglycol (PEG)	River and waste water	C$_{18}$-SPE	+	[40][b]	[40,81][b]	–	[81,88][a] [79][ab]	–	–

Polypropyleneglycol (PPG)	Waste and river water	C$_{18}$-SPE	+	[40]b	[40,81,135]b	—	—	—	—
Carboxylated PEGs (mono- and di-carboxylated)	Waste, sea, river, ground water	GCB	+	[86]b	—	—	—	[86]b	—
	Waste water	C$_{18}$-, LiChrolut EN, GCB-SPE	+	—	[99]a	[99]a	—	[99]a	—
	River water	C$_{18}$-SPE	+	—	—	[81]b [88]a	[81]b [88]a	—	—
Carbonylic PPG compounds	Waste water	C$_{18}$-SPE	+	—	[135]a	—	[79]a (PIS)	—	—
Carboxylic compounds (fluorinated)	Waste water	C$_{18}$-SPE	+	—	[104]b	—	[104]b	—	—
Carboxylated APEOs (mono- and di-carboxylated)	Standard	—	+/—	—	[72]a	—	[72]a	—	—
	Surface water	C$_{18}$-SPE	+	—	[105]a	—	—	—	—
	Waste water	Carbograph-SPE	+	[146]b	—	[146]b	—	[146]b	—
Carboxylated LAS (mono- and di-carboxylated)	Standard	—	—	[72]b	[72]b	—	[72]b	—	—
	Sea water	C$_{18}$-, SAX-SPE	—	[53]b	—	[53]b	—	[53]b	—

TABLE 19.1 *(continued)*

Compound	Sample/matrix	Isolation	Mode	Determination		Identification		Quantification	
				LC	FIA	LC	FIA	LC	FIA
	Waste water	C_{18}-SPE	–	[135][b]	[135][ab]	–	[135][b]	–	–
Biogenic surfactants									
Rhamanolipides	Cultured	TLC (prep.)	+	[80][b]	[80][b]	–	[80][b]	–	–
Cyclic sorphose lipids	Cultured	TLC (prep.)	+	[80][b]	[80][b]	–	[80][b]	–	–

[1] MS method is indicated by superscript letters as follows: (a) APCI, (b) ESI, (c) MALDI, (d) FT-MS, (e) FAB, (f) ToF-SIMS, (g) FD. PIS, parent ion scan.

decomposition, therefore time-consuming and discriminating derivatization steps are necessary. This pretreatment step is essential if GC-MS is used, the most frequently applied separation technique with substance specific detection in environmental analysis. The surfactants, discharged almost completely into the environment, show relatively low acute toxicity and no tendency towards bioaccumulation. Therefore producers and appliers were not urged to clear up their metabolic pathways and final fate. The analytical methods available today allow to follow up these pollutants in the biochemical degradation process in the environment. So the environmental relevance of these compounds and their metabolites could be confirmed which lead to intensified research on this topic, represented in an increased number [59,65] of results shown in Table 19.1.

With improved analytical methods available today the mixture analytical approach applying MS-MS or MS^n without any separation of the surfactants and/or their metabolites from polar matrix components in the time-saving flow injection analysis mode (FIA) is published in literature very often. With this technique it is possible to identify and quantify the largest part of these non-volatile pollutants in environmental samples without time-consuming LC separations. Selective elution after SPE concentration may help to overcome some problems arising from mixture analysis. However, co-eluting isomeric compounds from complex environmental matrices identical in their m/z ratios may lead to mixed CID spectra generated from several compounds with different structures. These spectra cannot be interpreted, and therefore LC separation is absolutely necessary under these circumstances. The same problems may rise during quantitative FIA determinations, if quantification is not done in characteristic scan modes using substance-specific ion mass traces of daughter or parent ions or neutral loss scans. This requires MS-MS or MS^n in the FIA mode, otherwise the application of LC is essential, too.

The prediction [59] that 'MS detection is more and more applied as substance-specific determination in the form of routine methods in environmental analysis as well as in other fields' has proved true. With progressive development of the analytical equipment the possibilities in detection, identification and quantification will more and more improve. In future the FTICR-MS technique (fourier transform ion cyclotron resonance mass spectrometry) with its high resolution potential providing elemental compositions of parent and fragment ions will become one of the leading techniques, because of its potential to identify compounds in this way.

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Chapter 20

LC/MS interfacing systems in environmental analysis: application to polar pesticides

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CONTENTS

20.1 INTRODUCTION

In recent years, much attention has been devoted to the trace-level analysis of polar pesticides and related compounds in environmental matrices. These compounds are frequently omitted from surveillance analysis of pesticide residues because they cannot easily be identified and/or confirmed by gas chromatography/mass spectrometry (GC/MS) [1]. Thermal lability, low volatility, and high polarity are the three main reasons for the failure of direct GC/MS determination. In such situations, liquid chromatography (LC) generally is the most appropriate separation method, and direct LC/MS analysis is an effective way to obtain both qualitative and quantitative information (for reviews, see [2,3]). The sample polarity and molecular mass ranges amenable to individual interfaces are summarised in Fig. 20.1.

Since even low concentrations of modern pesticides and their degradation products can cause a serious threat to life, they have to be monitored at trace levels of, typically, 0.1 μg/l
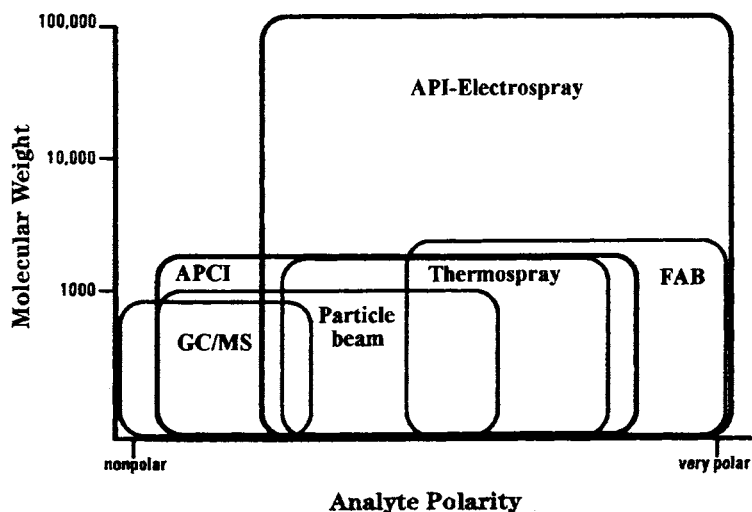
Fig. 20.1. Comparison of current LC/MS interfaces and GC/MS. PB, particle beam; TSP, thermospray; ESP, electrospray, APCI, atmospheric pressure chemical ionisation; cf-FAB, continuous-flow fast atom bombardment.

for tap water, 0.1–1 µg/l for surface water, and 0.1–1 mg/kg for food and foodstuff. Most LC-based methods use ultraviolet (UV), fluorescence or electrochemical detection, which is occasionally combined with post-column treatment, e.g. derivatisation. However, MS has the advantage over these conventional detectors that it can provide information for confirmation or unambiguous identification. LC/MS of polar pesticides was previously reviewed in [4–6].

Although the first coupling of LC to MS was reported over 25 years ago, and several LC/MS interfaces were described in the course of time (see e.g. [7–12]), the technical difficulties involved in interfacing a liquid flow of up to several ml/min with the high vacuum of a mass spectrometer source prevented the widespread use of LC/MS in method development until about 12 years ago. Nowadays, the practice of LC/MS has arguably become the single most widely publicised analytical technique [229]. The technique has developed rapidly as a commercially successful tool, fostered through the efforts of the mass spectrometry community rather than the chromatographers. These developments and the lower price of mass spectrometers in general have led to the situation that some MS instruments are now actually being sold as integrated detectors for liquid chromatography [11], which is often let working overnight in fully automated set-ups. LC/MS is widely applied in biomedical and biochemical research, and pharmaceutical product quality control, most often for qualitative purposes. LC/MS procedures for environmental analysis, often directed at quantitative analysis of target compounds or the identification of unknowns, have already found their place in many laboratories, as is demonstrated by several reviews on the subject [4,6,10–21].

In this chapter, major developments in the application of LC/MS in environmental analysis, particularly the determination of polar pesticides and related compounds, will be outlined for three interfacing techniques: thermospray (TSP), particle beam (PB), and

atmospheric pressure ionisation ((API): electrospray (ESP) and atmospheric pressure chemical ionisation (APCI)). In the last five years, no studies have been published on direct liquid introduction [10,22] and moving belt interfacing [23,24]; these techniques will therefore not be discussed. Continuous-flow fast atom bombardment (cf-FAB) will not be discussed either, because pesticide analysis is only rarely performed using this method. API and PB techniques are now well established as the cornerstones for future interface developments, while TSP has been largely superseded by APCI [11,25]. Even though TSP can be considered as obsolete, from a scientific point of view, in terms of performance it is close to APCI and applications presented for TSP may be of value also for APCI users.

From among the vast number of pesticides presently in use, attention will be focused on phenylurea herbicides, triazines, carbamates, organophosphorus pesticides, chlorinated phenoxyacetic acids and quaternary ammonium salts. Most of these compound classes cannot easily be analysed by means of GC and, moreover, all of them have successfully been analysed by LC with a variety of detectors. Analyte detectability and the possibility of obtaining structural information for the identification of target and non-target analytes will be included in the discussion.

20.2 LC/MS INTERFACES

20.2.1 Thermospray

The thermospray interface, as it was originally developed by Vestal and co-workers [26], uses heated nebulisation of the LC column effluent and extra pump capacity for effective desolvation. The TSP interface, which is schematically depicted in Fig. 20.2A, consists of a resistively heated capillary (the 'vaporiser') which generates an aerosol from the effluent. The aerosol sprays into a desolvation chamber, which is maintained at fore-vacuum pressure by a high capacity pump. The aerosol spray is directed perpendicular to the mass spectrometer entrance; the desolvation chamber and the mass spectrometer are separated by one or more skimmers and they are differentially pumped. Any ions in the aerosol may be forced into the mass spectrometer by a repeller electrode, which is commonly placed opposite the skimmer. The TSP can create sufficient desolvation, i.e. MS pressures can be kept below 10^{-4} Torr for conventional-size LC.

The process of vaporisation is schematically depicted in Fig. 20.2B. Although the precise mechanism of ion formation is still the subject of some controversy [27], a summarised view is given here. Due to the combined action of heat and low pressure, the column effluent breaks up into large droplets, which in turn evaporate volatile constituents, particularly solvent molecules. Evaporation proceeds inside the desolvation chamber to give fully desolvated analyte molecules and primary ions; these primary ions are formed by ion evaporation [27]. Effectively, ion evaporation is achieved by the addition of a volatile buffer salt, typically ammonium acetate, to the eluent. Ionic analytes, e.g. quaternary ammonium salts, do not always require the addition of a salt, because they may undergo ion evaporation themselves. The primary ions become available for gas-phase reactions with the neutral analyte molecules. The mode of ionisation which involves ion evaporation and subsequent gas phase reactions

(A)
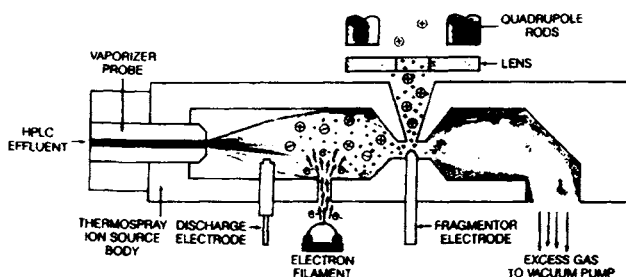
(B)
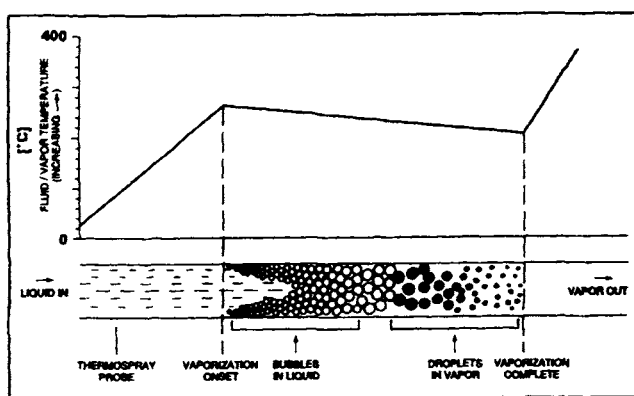
Fig. 20.2. (A) Schematic diagram of a thermospray interface [129]. (B) Temperature profile over the length of the vaporiser and schematic illustration of the thermospray nebulisation mechanism.

is generally termed 'buffer ionisation' TSP. Alternatively, proton transfer reactions may be stimulated by using a filament or discharge electrode; this is generally termed 'filament assisted TSP' or 'discharge assisted TSP', respectively. Assisted TSP effectively creates chemical ionisation conditions inside the desolvation chamber, with the eluent constituents as the reaction gas. The buffer salt ions, e.g. NH_4^+, produced by buffer ionisation TSP, are generally less reactive than reactants from the eluent produced in either of the other modes, e.g. $CH_3OH_2^+$. The resultant charged constituents of the aerosol may then be transferred to the mass separation system by the combined action of the high vacuum and the repeller electrode.

Ionisation by evaporation or by gas-phase reactions, 'soft ionisation', produces ions with a minimum of internal energy and thus leads to a low abundance of ion fragmentation processes. Enlargement of the mean ion kinetic energy through high repeller voltages, may be used to enforce dissociative collisions. This form of collision induced dissociation (CID) is of limited use for structure confirmation in TSP; higher repeller voltages are not compatible with optimal transmission and sensitivity is lost [28]. In general, TSP mass

spectra show abundant quasi-molecular ions, $[M + H]^+$, $M^{-\circ}$ or $[M - H]^-$, of the analytes.

The introduction of the TSP interface provided a major breakthrough in the application of LC/MS to analytical problems, enabling compound analysis at conventional LC flow rates, and with regard to good sensitivity. The interface is simple to use, and robust, and until the mid-nineties was commercially available for most MS instruments, which made it the most widely used LC/MS interface. TSP mass spectra commonly yield little structural information and they are, therefore, of limited use for the identification of unknown compounds (Fig. 20.3c).

Since identification capability is often highly desirable in environmental analysis, TSP–MS is mainly used for target compound analysis. More recently, there has been a trend to try and obtain more structural information from LC/TSP–MS/MS rather than LC/TSP–MS. The use of tandem-MS (MS/MS) provides an elegant means of obtaining structural information, albeit at the cost of an increase in complexity of the method [29–38]. A schematic representation of a typical triple quadrupole MS/MS operation is shown in Fig. 20.4 (top). The loss of sensitivity, which is to be expected from a decreased ion transmission in MS/MS, generally appears to be compensated by an enhanced signal-to-noise ratio (selectivity).

Actually, only few authors justify their use of MS/MS quantification by providing quantitative data [29]. The MS/MS approach is also highly useful for the identification of unknown pesticide metabolites formed during degradation processes.

Alternatively, a tentative confirmation of analytes with the help of an exact mass determination can in principle be accomplished by coupling the TSP interface to a magnetic sector mass spectrometer [39–42] or by using a modified quadrupole mass spectrometer [43], but such experiments have not yet been applied to pesticides. It should be noted that exact mass measurement under TSP conditions requires the addition of an internal standard, and elaborate data handling. Also, because of the fact that higher resolution implies less sensitivity, TSP with exact mass determination is not too attractive for routine application.

With the advent of APCI, the TSP became rapidly more or less obsolete and only a limited number of publications concerning TSP has appeared since 1995. Among the distinct advantages of APCI is the possibility to decouple MS from the interface, which is operated at atmospheric pressure: in this way the operating conditions can be optimised for both the LC separation and MS detection. The instrumentation is rugged because the majority of the analytes and solvents remains in the atmospheric chamber and is vented away without degrading the MS performance. Also, liquid droplets are desolvated more efficiently at atmospheric than at reduced pressures, a result of more efficient heat transfer occurring at the higher pressure. Finally, ions sampled to the MS undergo a free jet expansion, which causes adiabatic cooling and helps maintain integrity of labile compounds and non-covalent complexes for mass analysis. Furthermore, the electrostatic potential in this expansion region can be controlled and in-source CID (for more details, cf. Section 2.3) can be achieved, which helps to provide structural information.

One option open to laboratories with limited budgets is retrofitting an existing TSP–MS instrument with either an ESP or an APCI interface for ca. 20–30% of the price of a new API–MS [44–47].

Fig. 20.3. Comparison of mass spectra of aldicarb obtained by (a) 100 ng injected using PB–EI, (b) 100 ng injected using PB–methane PCI, (c) 20 ng injected using TSP and (d) 100 pg injected using ESP at a capillary voltage of 90 V [15].

MS/CID/MS

| API ion source | Stage 1 | Stage 2 | Stage 3 |
| No separation | Mass separation | Fragmentation | Mass analysis |

LC/CID/MS

Stage 1	Stage 2	Stage 3
HPLC	API ion source	Mass analysis
LC separation	Fragmentation	Mass analysis

Fig. 20.4. Schematic diagram of in-source CID process in a single quadrupole MS (bottom) and in a triple quadrupole mass spectrometer (top), used to obtain product–ion scans (courtesy of Hewlett-Packard).

20.2.2 Particle beam

The introduction of the PB interface, originally known as the 'monodisperse aerosol generating interface for chromatography' (MAGIC-LC/MS) [48] enables the coupling of a wide range of LC separations to conventional EI and CI MS procedures. LC/PB–MS is nowadays mainly used for the identification of non-target compounds in real-world matrices. The interface is commercially available under various names, i.e. 'particle beam' (with concentric pneumatic nebuliser), 'thermabeam' (with thermally assisted pneumatic nebulisation) and 'universal interface' (with thermally assisted pneumatic nebulisation and additional membrane separation). Of all LC/MS interfacing methods, LC/PB–MS comes closest to GC/MS; the PB interface is principally a momentum separator and as such it is derived from the jet-type GC/MS interface (used with packed columns [49]).

A scheme of a PB interface is shown in Fig. 20.5. Desolvation occurs in steps by leading the column effluent through several differentially pumped chambers. At the exit of the LC column a liquid jet is generated by pumping the effluent through a small (25 μm) orifice into a low vacuum (200 Torr) desolvation chamber. The liquid jet is then dispersed into droplets with a nearly uniform size distribution, by the action of a helium flow. Part of the volatile constituents of the effluent, mainly the solvent, evaporates from the aerosol droplets; this evaporation is supported by heating the desolvation chamber (the pressure allowing heat exchange with the droplets). The aerosol is then forced through a nozzle, into a second vacuum chamber (at about 10 Torr). Both the dimension of the nozzle and the pressure drop result in a supersonic expansion, which is characterised by a laminar flow pattern in which relatively heavy particles move preferentially near the axis. These

	NEBULIZER	DESOLVATION CHAMBER	MOMENTUM SEPARATOR	PB/MS INLET	MS ENGINE
PRESSURE	<30 BAR (BACK PRESSURE)	200 TORR	STAGE 1 < 10 TORR STAGE 2 < 0.9 TORR	*	*
TEMPERATURE	AMBIENT	~55°C	AMBIENT	AMBIENT	200-350°C

* VARIES WIDELY DEPENDING ON IONIZATION MODE
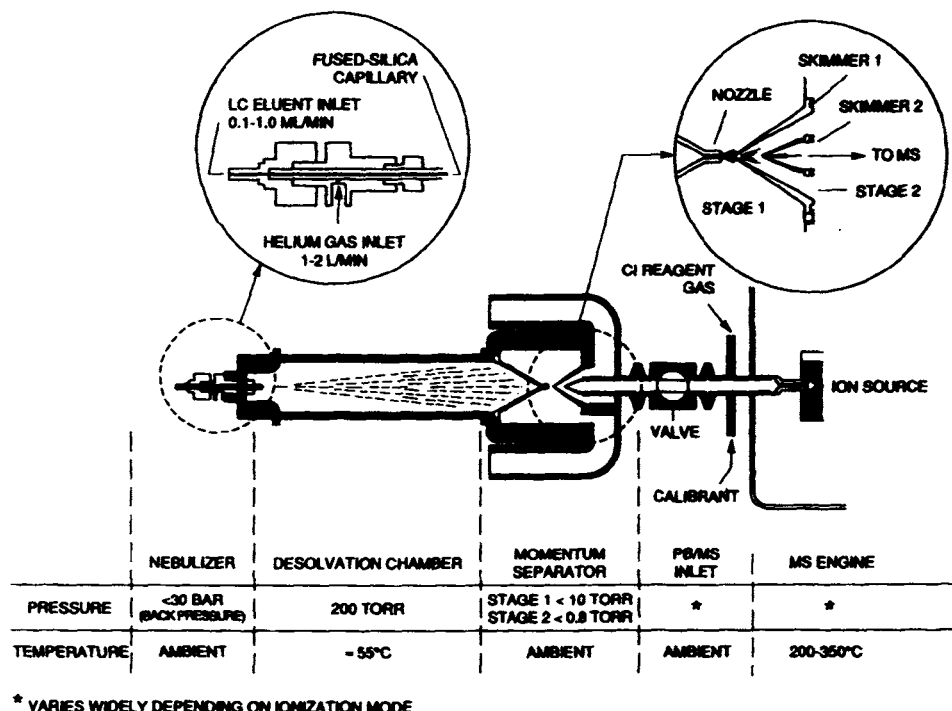
Fig. 20.5. Schematic diagram of a particle beam interface (Hewlett-Packard 59980B).

heavy particles contain more of the analyte than the lighter particles and an enrichment is effected by selective cutting of the centre of the flow pattern with the help of a skimmer. Another skimmer is used to increase the efficiency of the momentum separation and the resulting particle beam is then led through another vacuum chamber (pressure about 1 Torr) into the mass spectrometer ion source. Inside the source, collision with the source wall breaks up the non-covalent bonds in the particles and the resulting single molecules can be ionised. The operation of a PB interface is based upon vaporisation of solute aggregates over a hot surface, and the analytes have to be sufficiently thermostable to survive this process. For a majority of the analytes of environmental interest, the heat transfer is so rapid that thermal decomposition is rarely observed.

The flow rates that can be accommodated by the PB interface are generally compatible with conventional-size LC; they lie between 0.1 and 1.0 ml/min [18], with the maximum attainable flow rate being lower for higher eluent water content. The limiting values are the result of the flow rates required to form a stable liquid jet, on both extremes of the flow rate regime, and the limited capacity to desolvate the aerosol, in the high flow region. LC/PB–MS mostly yields relevant structural information from the generated EI spectra. Besides, the technique is well suited for thermolabile compounds, because the amount of heat applied during desolvation is usually limited; this even holds for the modified PB interfaces, the 'thermabeam' [50] and the 'universal interface' [51]. A principal advantage of PB over all other LC/MS interfacing methods is that it can be coupled to any mass spectrometer, without (or with minor) modification.

Two closely related drawbacks of LC/PB–MS are the low sensitivity and the non-linearity of the response. The low sensitivity is due to the low analyte transmission efficiency of the interface [52], which typically lies between 0.5% and 1%. The non-linearity of the signal intensity in LC/PB–MS at low analyte concentrations was first reported by Bellar and co-workers [53]. This phenomenon has been the subject of detailed studies [52,54–56], but so far the results remain inconsistent. The analyte transmission efficiency and the non-linearity of the response are commonly thought to be related to the efficiency of the formation of solid particles in the evaporation process [52,56], but there is no compelling evidence to support this. Compounds such as malic acid [57] and ammonium acetate [58] have been added to the LC eluent, to improve particle formation at low analyte concentrations; the rationalisation behind this approach is that a compound-specific or non-specific 'carrier effect' can be achieved. However, such enhancement is not always observed and it appears to differ for each analyte. As an alternative to solvent additives, glow discharge heating in the nebulisation stage of PB was tested for the improvement of the transport efficiency [59]. The effect of the discharge on sensitivity is comparable to that of the addition of ammonium acetate, but a combination of additive and glow discharge did not perform better than either of the two separate modifications. In view of the unresolved non-linearity of the response, it was proposed that a calibration method with coeluting isotopically labelled internal standards is most reliable for real-world environmental samples [55,56].

The PB interface has also been coupled to an ion trap mass spectrometer (ITMS) [60] and to a magnetic sector instrument [16]. With the ITMS, a third-stage momentum separation was added to the interface to improve desolvation. This is an attractive approach, because of the multiple MS/MS options and the high sensitivity, through ion storage capabilities. The magnetic sector mass spectrometer was used to aid structural elucidation and confirm molecular formula by high-resolution EI data. The combination of PB and these types of mass spectrometer have not yet systematically been applied to pesticide analysis. A general review on LC/PB–MS, covering instrumentation, studies on the PB mechanism and selected applications, was published by Creaser and Stygall [18].

General acceptance of the PB interface in environmental analysis will require considerably improved detection limits; the present values, which typically are in the 10–500 ng range, will often not be sufficiently low. A viable approach to overcome this problem for the analysis of water samples is to use on-line trace enrichment prior to the LC separation, with direct introduction of the LC column effluent into the PB interface (Fig. 20.6) (see e.g. [61–63]). Since the on-line trace-enrichment step is carried out in such a way that it is independent of the LC/MS interfacing, it can be used to advantage in essentially all LC/MS procedures involving the analysis of aqueous samples or sample extracts.

One possibility to optimise PB–MS performance is an application of statistical experimental design [64]. The results of the quoted study show that commonly used flow rates, temperatures of the desolvation chamber, the composition of the mobile phase and the concentrations of mobile phase additives are in most cases far from the optimum conditions (e.g., optimum flow rate 0.2 ml/min instead of 0.4 ml/min recommended by most manufacturers).

In spite of the compatibility of PB–MS with conventional-size LC, several promising studies have appeared on micro-flow LC/PB–MS [65–69]. The delivery of micro-LC flows as low as 1 μl/min to a PB interface prompted the development of a modified nebuliser,
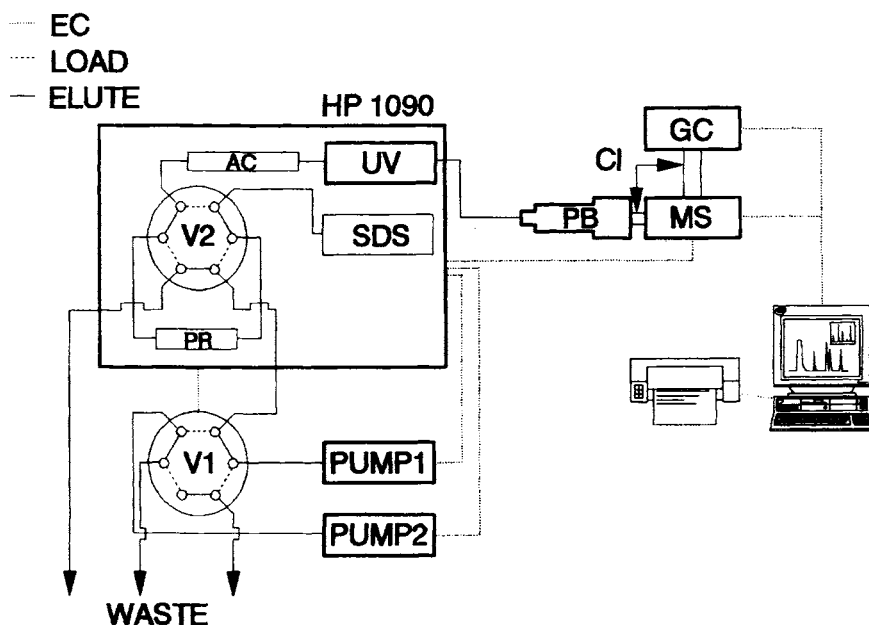
Fig. 20.6. Set-up for automated on-line SPE/LC/PB–MS. HP 1090, liquid chromatograph; V1, V2, automatic six-port switching valves; LOAD/ELUTE, positions of V1 and V2; EC, electronic connections; PUMP 1, PUMP 2, preparative LC pumps; PR, precolumn; AC, analytical column; SDS, solvent delivery system of liquid chromatograph; PB, particle beam interface; GC, gas chromatograph; CI, chemical ionisation reagent gas inlets; MS, mass spectrometer. 100–250 ml water sample is first enriched on precolumn packed with specific sorbent and next, after switching the six-port valve V2 into ELUTE position, eluted onto analytical column by LC effluent gradient [63].

which was shown to provide the usual PB–MS performance at lower vacuum contamination rates [65]. A significant reduction in solvent consumption and better analyte responses for mobile phases with higher water content were reported as advantages over 'conventional' PB systems. Using the modified system, instrument detection limits comparable to those obtained by API–MS were reported [68,70]. In the work by Kientz et al. [69], an eluent-jet interface was used for coupling packed microcapillary columns to electron-ionisation MS. The interface combines thermal effluent nebulisation with a conventional jet-separator, as applied in the past in packed-column GC/MS. A SIM detection limit of 50 pg was reported for caffeine. The system is currently modified to implement a more advanced PB interface [71].

The most recent patents dealing with improvements in PB interfaces appeared in 1993 (introduction of Waters Integrity LC/PB–MS) and quite a number of publications on PB–MS keeps appearing. Despite this, the sales efforts of most instrument manufacturers were refocused on the more attractive API technology. Improvements in the sensitivity of the PB–MS is the major issue addressed in new interface designs [67,69,71]. Effective sample concentration techniques, either off-line [70] or on-line [61] and, most probably, the micro-flow technology by Cappiello et al. [67] may well bring desired analyte detectability.

Although PB–MS is nowadays overshadowed by an abundance of API instrumental developments and applications, it will certainly continue to play an important role in LC/MS interfacing [12,67,72].

20.2.3 Atmospheric pressure ionisation

In the field of LC/MS coupling, most current interest is in the use of atmospheric pressure ionisation (API) methods [11,12,20,21,73,74]. The present API technology in fact comprises of two different interfaces for LC/MS, based on electrospray ionisation (ESP) and atmospheric-pressure chemical ionisation (APCI). In an API–MS system (Fig. 20.7, also see Fig. 20.10 below), the ion source region (located outside the mass spectrometer, at ambient pressure) is separated from the high-vacuum mass analyser region by a small ion-sampling orifice. This orifice must be large enough to permit the introduction of a significant proportion of the ions from the atmospheric pressure region into the high-vacuum region. The LC column effluent is sprayed in the vicinity of the orifice, so that free-jet expansion and concomitant adiabatic cooling occur. Nebulisation is either performed pneumatically (in APCI), by means of the action of a strong electrical field (in ESP), or by a combination of both (ionspray or pneumatically assisted ESP). The formation of a spray is effected by applying heat [75], a coaxial nebuliser gas stream [76], an electrostatic potential, ultrasonic vibration of the capillary [77], or a combination of these. Among other sample introduction methods, primarily used in APCI, are atmospheric-pressure spray and sonic spray [78,79]. The free-jet cooling contributes to the formation of large clusters of analyte and solvent molecules, which are bound by Van der Waals forces. Desolvation of the clusters is effected by collisions; the collision rate is enhanced either by leading the ions through a gas curtain or by accelerating them through an electric field gradient. Ionisation is effected by applying a high voltage over the spray (electrospray, ESP) or by using a combination of a heated capillary and, e.g., a corona
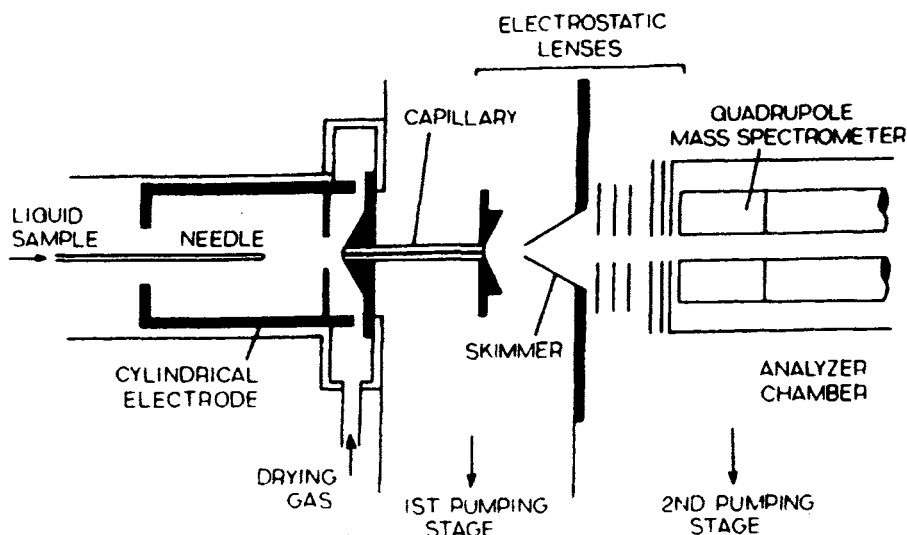
Fig. 20.7. Schematic diagram of the electrospray interface [84].

discharge (atmospheric pressure chemical ionisation, APCI). Ions are produced from the evaporating droplets, either by gas-phase ion-molecule reactions initiated by electrons from a corona discharge (in APCI), or by the formation of microdroplets by solvent evaporation and repetitive electrohydrodynamic explosions and the desorption, evaporation or soft desolvation of ions from these droplets into the gas phase (in ESP). The ionisation process in ESP is very soft, generating $[M + H]^+$ or $[M - H]^-$ ions for even the most unstable and non-volatile compounds. In APCI, the ionisation process puts the analyte into the gas phase, making it unsuitable for truly non-volatile and thermally unstable compounds. In ESP droplet formation and charging take place simultaneously, while in APCI droplets are formed prior to ionisation. Initially, the main distinction between ESP and APCI was in terms of the eluent flow rates and the molecular weight ranges that can be handled: low flow rates (up to 20 μl/min) and a high molecular mass range in ESP as compared to high flow rates (up to 2 ml/min) and a low molecular mass range in APCI. Nowadays, ESP can be performed at higher flow rates, by directing a gas flow into the effluent stream [80] (designated 'pneumatically assisted ESP', 'high-flow ESP' [81], 'turbo-ionspray', 'thermally assisted ESP', 'ultrasonically assisted ESP', 'multichannel ESP' or 'ionspray', ISP. This nomenclature, mainly dictated by patents, distracts attention from the fundamental similarities between the various approaches. In this review, the term 'electrospray' is used throughout, eventually with 'pneumatically-assisted' as adjective. The term 'ionspray' is kept for historical reasons. Generally, in API systems, quite some amounts of gas for nebulisation, auxiliary gas, and in some systems countercurrent gas, are required. This requirement can be as high as 600 l/h, which requires at least a bottle of pressurised nitrogen of high purity per 24 h of operation.

In all API sources until 1995, the spray device is in axial position, or only slightly off-axis, relative to the sampling orifice or capillary. Recent interface designs, e.g. the orthogonally positioned spray device [82,83] (Fig. 20.8) and the so-called Z-spray (Fig. 20.9), allow introduction of a flow of several ml/min. The system [82] can be used for both ESP and APCI. The orthogonal position of the sprayer significantly reduces the contamination
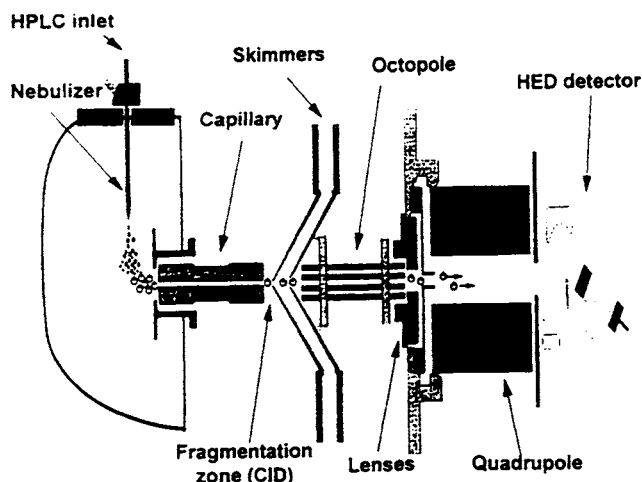
Fig. 20.8. Schematic diagram of the Hewlett-Packard orthogonal electrospray system.
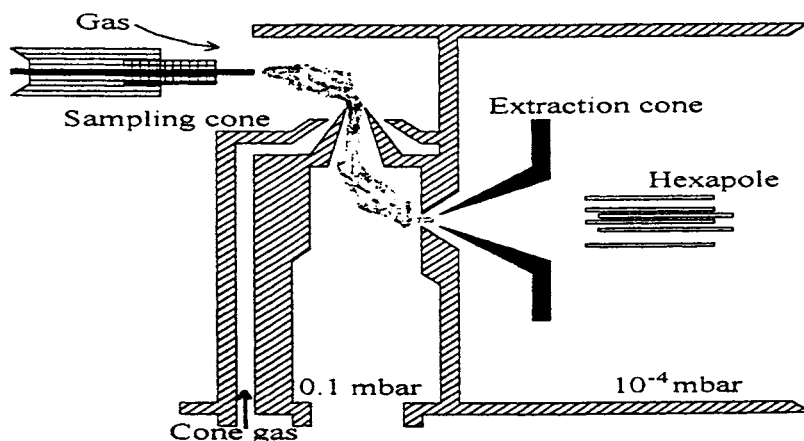
Fig. 20.9. Schematic diagram of the Micromass Z-spray electrospray source.

of the sampling orifice. It has various advantages, such as more intense signals, reduction of ion source contamination and compatibility with flow rates up to 4.4 ml/min without experimental difficulties. An important additional feature of the system is that the control, data acquisition and data handling in LC/MS operation is fully integrated in the software of the LC and DAD UV detector. As such, the system can be considered as a powerful additional detector for LC, the operation of which does not significantly differ from UV detection. In the Micromass Z-spray, ions are orthogonally extracted from the API source into a pumped chamber, and subsequently drawn into the high vacuum chamber for mass analysis. This system should allow the use of phosphate buffers in the mobile phase. Since these developments are very recent, proper evaluation of the performance and claims of the manufacturers is not yet possible.

In a typical ESP experiment, for which the set-up is shown in Fig. 20.7, sample solutions enter the spray chamber at a flow rate of 1–10 μl/min. The liquid is electrosprayed from the tip of a hypodermic needle and the droplets formed are further dispersed by means of a counter current stream of heated nitrogen gas of ca. 150 ml/min. The solvent vapour from the rapidly evaporating droplets is swept away by this so-called bath gas and the ions formed are transported through an orifice or a capillary into a first vacuum chamber, where a supersonic expansion occurs. The core of the expansion is sampled by a skimmer, kept at about −20 V, and transported into the mass analyser region [84]. Competition between the coulomb repulsion of the charged droplets and the surface tension of the liquid plays a major role in the formation of the ESP aerosol, but the actual mechanism of ESP ionisation is still a matter of discussion [10,85]. The major drawback of LC/ESP–MS is that the maximum allowable flow rate is in the order of 10 μl/min, with lower flow rates giving better performance. As regards the compatibility to conventional size-LC, the ESP interface is often quoted to be a 'concentration-sensitive' device [86]; this would imply that splitting of the LC eluent flow does not cause a loss of sensitivity.

The ISP interface was originally introduced to enhance the ion evaporation of the ESP. The main advantage of the ISP interface, developed by Bruins et al. [80], over the ESP interface is the tolerance of higher liquid flow rates. In the ISP interface (Fig. 20.10), the
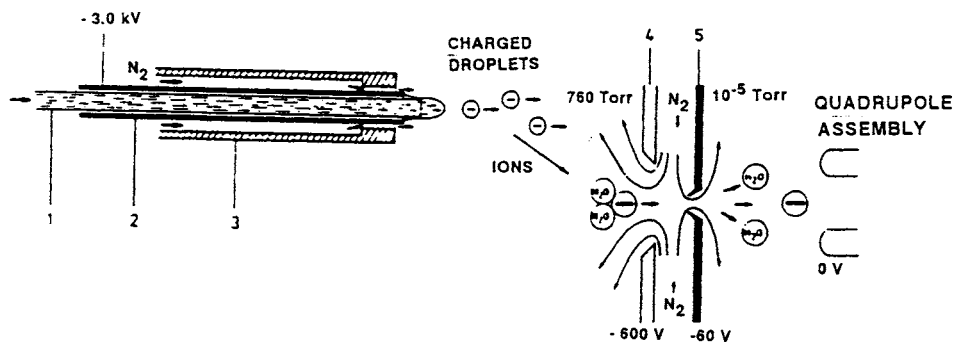
Fig. 20.10. Schematic diagram of (left) ion spray (ISP) interface and (right) atmospheric pressure ion (API) source (not drawn to scale) with nitrogen gas curtain: (1) 50 μm i.d. fused-silica capillary; (2) 0.20 mm i.d. stainless steel capillary; (3) 0.8 mm i.d. Teflon tube with narrow bore insert; (4) ion focusing lens, serving as counter electrode for ion spray; (5) orifice holding plate with 100 μm i.d. conical orifice [80].

electrospraying process is assisted by coaxial pneumatic nebulisation of the LC column effluent. ISP has even been coupled successfully to a benchtop-size mass spectrometer [87]. Flow rates of 40–50 μl/min, which are compatible with 1 mm i.d. LC columns, can be accommodated. ISP shows improved performance over TSP with thermolabile ionic compounds, because ISP operates at room temperature, while heat has to be applied with TSP. The introduction of a 'liquid shield' device, which protects the ion sampling orifice region of the system from droplets formed in the spray process, and heating of the capillary interface allow the use of conventional LC flow rates of 1–2 ml/min [88]. Such a device is incorporated in the sources available on the API-100/300 series from Perkin-Elmer Sciex. Although ESP is used mainly for the analysis of large molecules (proteins, peptides), it has also been extensively used, in both positive- and negative-ion modes, for the analysis of low-molecular weight compounds in environmental samples (see e.g. [89–92]).

As the name suggests, APCI involves gas-phase ion–molecule reactions, which cause the ionisation of analyte molecules under atmospheric pressure conditions. In APCI, both heat and pneumatic nebulisation are applied to evaporate the sample solution and to obtain an effluent spray (Fig. 20.11) [93]. Under these conditions, reversed-phase LC eluent flows
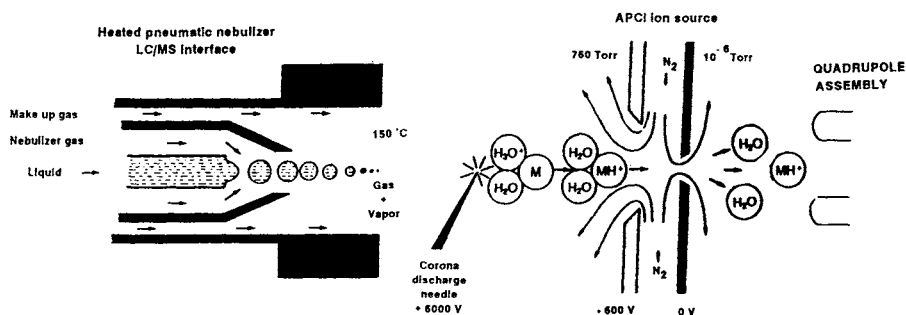
Fig. 20.11. Schematic diagram of a heated pneumatic nebuliser (APCI) interface [7].

of 0.1–2.0 ml/min can be handled. Reactant ion formation is achieved by the introduction of electrons, either from a corona discharge or from a ^{63}Ni foil. The discharge, established at the tip of a needle which is held at a high voltage (3–6 kV), is routinely used in combination with heated pneumatic nebulisation. In analogy to TSP, ion evaporation can potentially also contribute to reactant ion formation. Both electron mediated ionisation and ion evaporation are very mild ionisation processes which readily provide unfragmented quasi-molecular ions. The molecular weight information and the information obtained from in-source CID make it possible to use APCI–MS for identification purposes.

In the last few years, API-based methods have been further developed. APCI has been interfaced to MS/MS for the study of complex solids with a pyrolysis module [94,95]. Although API–MS was first developed with quadrupole mass spectrometers, it has successfully been coupled to an ITMS [96]. A major breakthrough in the field of coupling API to ITMS is the recent commercial introduction of two ion-trap based LC/MS instruments with an API source for ESP and APCI (Finnigan MAT LCQ and Bruker/Hewlett-Packard ESQUIRE). At present, there is only a limited number of publications available from independent laboratories; therefore, it is too early to evaluate the performance of these systems. Given the significantly reduced cost of an ion-trap MS/MS system compared to a triple-quadrupole system for MS/MS, extensive use of ion-trap instruments may be envisaged. The ion traps allow a stepwise breakdown of precursor ions in multiple stages of MS/MS, which facilitates structural elucidation of complex compounds. Preliminary results in quantitative analysis indicate that 5–10 times worse detection limits are achieved in selective reaction monitoring with an ion-trap system compared to a triple-quadrupole [97]. API was coupled also to a magnetic sector instrument [98,99,100–107].

Coupling of API to triple-quadrupole MS is currently the most popular set-up to be found in research and routine laboratories. However, the interpretation of the MS/MS product-ion mass spectra acquired on ESP-triple-quadrupole instruments is greatly hampered by the lack of knowledge on the charge state of the product ions. This has led to the implementation of ESP on instruments capable of efficient CID in combination with a high-resolution instrument, i.e. Fourier-transform ion-cyclotron resonance instruments [108]. Coupling of an ESP ion source to an FT-ICR-MS instrument has been studied by several groups of researchers (for a review, cf. [11]). The incentives in this research is the possibility of (ultra)high-resolution measurements, the improvements of detection limits by signal averaging, and the efficient tandem mass spectrometry potential. Commercial ESP interfaces for FT-ICR–MS instruments are available from several manufacturers [109,110].

API sources have also been coupled to time-of-flight (TOF) and MALDI–TOF instruments (for reviews see [11,111,112]). Here, especially the developments in coupling APCI to reflectron TOF analysers may be of interest for environmental analysis, since the instrumentation allows to achieve resolution as high as 6000 and accurate mass measurements of relatively small molecules [113,114].

An atmospheric pressure radio frequency plasma source, which operates with a variety of buffer gases, has been developed as an ionisation method for organic samples introduced into an API–MS by liquid injection [115]. Finally, microwave sources have been used to ionise organic molecules at atmospheric pressure [116]. API sources to be fitted onto TSP ion sources have also been described [44–46].

20.2.4 In-source CID

Generally, all API techniques provide soft ionisation in which little structural informa- tion is obtained directly. However, the application of an appropriate voltage difference between two regions of an API source, i.e. between the nozzle and the skimmer or between the skimmer and an octapole ion guide, generally induces fragmentation of the primarily formed ions; this mode of operation is termed in-source CID, pre-analyser CID or cone voltage fragmentation (CVF). A typical example of enhancement of structural information by changing the in-source CID voltage is shown in Fig. 20.12A,B. Considerable structural information can be obtained at high potential differences between nozzle and skimmer. This is of vital importance in environmental analyses because the soft ion evaporation process fails to produce the fragment ions necessary for confirmation of compounds of environmental interest. Obviously, the interpretation of such spectra can be difficult for unknowns, because the various fragment ions may have different charge states. Voyksner and Pack [117] as well as several others [118–120] demonstrated that in-source CID can also be achieved for small molecules and may be used in structure elucidation. They found that mass spectra of compounds like aldicarb, propoxur, carbofuran and cloxacillin obtained with a 30–50 V potential difference between nozzle and skimmer closely resem- ble those obtained in conventional (triple quadrupole) CID at 30 V collision energy. Good product ion yields were achieved with minimum losses in the overall ion current. Frag- mentation by in-source CID is sometimes denoted as 'poor-men's MS/MS' [11].

Although in-source CID certainly is a useful technique, the method has distinct limita- tions. By means of in-source CID *all* ions present are subjected to CID. In CID in the collision cell of an MS/MS instrument, on the other hand, a selection of a particular precursor ion is performed first, thus leading to significant improvements in signal-to-
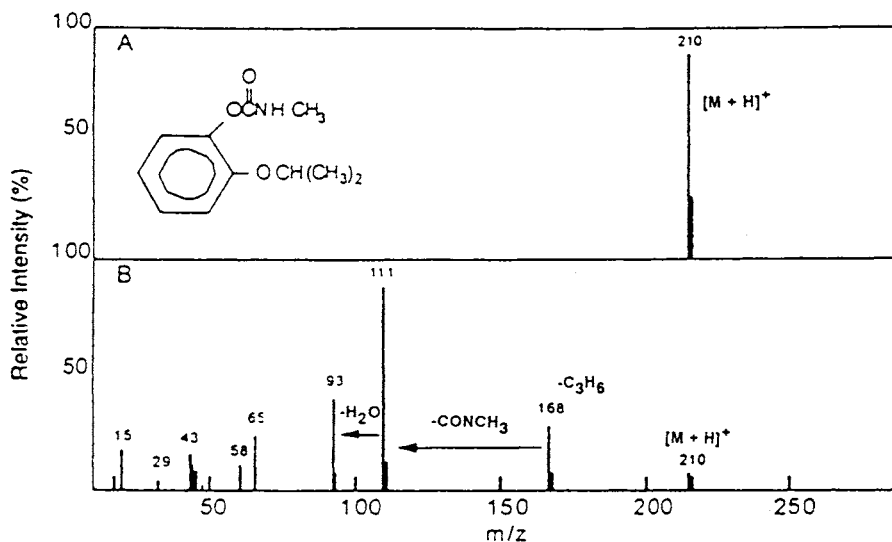
Fig. 20.12. ESP–MS spectra of propoxur at (A) capillary–skimmer potential difference of 25 V; (B) capillary–skimmer potential difference of 85 V. Spectra were obtained from 2 μg of propoxur in methanol/water at a flow rate of 2 μl/min [6].

noise ratios, which cannot be achieved by in-source CID. Obviously, because of the selection of the target ion in MS/MS, the CID process can be performed in a more controlled way. Few systematic studies have been reported on the comparison of in-source CID and CID in MS/MS. Most authors simply state that the fragmentation observed in in-source CID is similar (or identical) to that obtained in MS/MS, although significant differences in relative abundance of the various fragment ions are observed as well. With the new instruments, it is possible to 'toggle' the capillary voltage on alternate scans during the analysis under computer control. Thus one can acquire both molecular ion information (low CID voltage) and structural information (high CID voltage) in a single run. Because of the need to speed up analyte identification, attempts to build libraries of composite in-source CID spectra or MS/MS product ion spectra from protonated molecules were made by several researchers and manufacturers [121–123].

20.3 APPLICATIONS

20.3.1 Thermospray interface

Prior to the commercial breakthrough of API technology in the mid-nineties, TSP was the most frequently used interface in LC/MS of pesticides. This was due to its applicability to a wide range of compounds, its compatibility with most MS systems and its ruggedness. Selectivity enhancement has been achieved by adding volatile buffers, organic salts and halogenated organic modifiers to the LC eluent and by using normal-phase LC and post-column extraction. The identification potential has been improved by using TSP–MS/MS. As the technology matured, more attention was devoted to overall method development, and to attempts to automate the system and increase the sample throughput. Due to some similarities between TSP and APCI nebulisation, ion formation processes and application range knowledge from LC/TSP–MS method development is useful also for APCI users.

A wide range of pesticides has already been analysed using LC/TSP–MS. In general, when using ammonium acetate as an additive with reversed-phase LC, PI typically causes protonation (giving $[M + H]^+$ ions) and ammonium adduct formation (giving $[M + NH_4]^+$ ions), while NI causes deprotonation (leading to $[M - H]^-$ ions), anion attachment (resulting, e.g., in $[M + MeCO_2]^-$ ions) and electron capture (yielding $[M]^-$ ions). Occasionally, the primary ionisation process leads to fragment ion formation, mostly through the loss of a functional group.

Barceló et al. [124–128] reported studies on organophosphorus insecticides, chlorophenols, triazines, phenylureas, phenoxyacetic acids and carbamates. A variety of experimental conditions, e.g. employing different solvents, was used to obtain optimal detection. It was found that most of the pesticides investigated can be analysed straightforwardly by TSP. The selectivity of TSP using NI was demonstrated for several chlorophenols, which show no response in the PI mode. In general, signal intensities in the PI mode were about three orders of magnitude higher than those in the NI mode; lowest detectable amounts ranged from 5 to 50 ng.

It was shown by Voyksner et al. [129] that deterioration of the LC separation of several carbamate and phenylurea compounds by the TSP buffer salt could be prevented by post-column addition of the salt. Employing this, lowest detectable amounts in the PI mode varied from 1 ng for carbofuran to 80 ng for diuron; in the NI mode the sensitivity was 4–5

orders of magnitude lower. 0.1 M acetic acid was used for eluent acidification in the analysis of several sulfonylureas [130]. As the acidification resulted in a loss of sensitivity, the eluent was neutralised by post-column addition of ammonium hydroxide; this conveniently supplied the ammonium acetate needed for TSP ionisation. The sulfonylurea pesticides were determined in crops at the 50 μg/kg level, using SIM for quantification [131].

Carbamates, phenoxyacetic acids, chlorinated aliphatic acids, phenylurea herbicides and oxime fungicides were determined in environmental samples with TSP after off-line preconcentration [132]. Concentration was carried out by means of liquid–liquid or liquid–solid extraction of soil and water samples, respectively. The compounds were then separated by gradient elution, with ammonium acetate present in the LC eluent. Under full-scan conditions, the various compounds could be detected in liquid samples at the 0.1–1 μg/l level, with estimated detection limits in the 10 ng/l range. Chlorine-containing analytes, such as carbaryl and linuron, were determined in the NI mode.

Ammonium formate is sometimes preferred as an additive, in order to obtain structural information or to increase the scan range at the low mass end. A thorough study on the applicability of various additives in LC/TSP–MS was carried out by Voyksner et al. [133]. Ammonium acetate gave the best sensitivity, with detection limits of 20–60 ng for triazines and 30–300 ng for organophosphorus insecticides. In addition, the adduct ions generated with ammonium formate from triazines, phenylureas, chlorinated phenoxyacetic acids [134] and carbamates [135], provide complementary structural information which enables unambiguous molecular-weight assignment for unknown pesticides. Detection limits (using SIM) for simazine, atrazine and propazine were 5 μg/l while carbamates could be detected at the 50 μg/l level, under full-scan conditions.

The advantage of solvent (i.e., chloroacetonitrile) adduct ion formation was demonstrated for phenoxyacetic acid herbicides [136] and chlorophenols [137] in the NI mode. The addition of 2% chloroacetonitrile to reversed-phase eluents resulted in an $[M + Cl]^-$ base peak, thereby demonstrating abundant chloride attachment. Instrument detection limits for the chlorophenols used as test compounds, in the filament-on mode, were in the low-ng range. The same authors [138] gave an overview of the use of ammonium formate or acetate, non-polar solvents, or chloroacetonitrile to obtain structural information from TSP mass spectra of a variety of carbamates, chlorinated phenoxyacetic acids, chlorotriazines, organophosphorus insecticides and phenylurea herbicides.

A systematic study of 27 carbamates and their degradation products by FIA/TSP–MS was presented by Honing et al. [139]. It was observed that the addition of ammonium acetate, ammonium formate and nicotinic acid into the LC eluent suppresses fragmentation in spectra and favours adduct ion formation. This led to a, typically ten-fold, increase in sensitivity for PI detection of carbamates, whereas the 'non-carbamate-type' degradation products did not generate adduct ions. In terms of sensitivity, NI detection was found to be less favourable for all carbamates studied. Thermal degradation of methiocarb and its sulfone were observed by varying the vaporiser temperature.

Effects of various additives in the LC eluent on analyte sensitivity and selectivity in LC/TSP–MS were studied in detail by Vreeken et al. [140], for 55 pesticides. The use of discharge ionisation was generally preferred over the filament-on or buffer ionisation, because of lower detection limits and increasing structural information in the spectra obtained. Full-scan detection limits for 15 different classes of pesticides, were typically

in the range of 20–200 ng (Table 20.1), except for anilines and quaternary ammonium compounds.

The potential of TSP with non-polar, normal-phase LC solvents (n-hexane, cyclohexane and dichloromethane) for the analysis of pesticides has been evaluated [141]. In the PI mode, detection limits improved about 10-fold for all test compounds when using a normal-phase instead of a reverse-phase eluent. In the NI mode both types of eluent showed the same sensitivity for several chlorophenols. Using full-scan TSP–MS, detection limits for nine most commonly used groups of pesticides varied between 1 and 200 ng in both PI and NI (Table 20.1). This is in line with the results of a study on the determination of phenoxyacetic acids and chlorotriazines using cyclohexane as a LC eluent [142]; here, good sensitivity was obtained in the PI mode.

LC separation methods using non-volatile buffers and ion-pair reagents, which cannot be introduced into a TSP–MS, can be used if an on-line post-column extraction into a non-polar solvent is carried out, with subsequent phase separation in, e.g., a sandwich-type phase separator. In one study, the procedure was used to determine 2,4-D, 2,4,5-T and

TABLE 20.1

CALCULATED LIMITS OF DETECTION (LODs)[a] FOR VARIOUS GROUPS OF PESTICIDES IN LC/TSP-(FULL-SCAN)-MS [140]

Class of pesticides	LOD (ng) in					
	PI			NI		
	>200	20–200	<20	>200	20–200	<20
N-substituted amides		+		+		
Anilines	+			+		
Carbamates		+			+	
Thiocarbamates	+					+
Hydroxy-keto-lactones		+				+
Miscellaneous compounds[b]	+				+	
Organophosphorus compounds		+				+
Phenols	+					+
Phenoxy and carboxylic acids	+					+
Pyridine-like compounds		+				+
Quaternary ammonium compounds	+			+		
Thiocyanates		+		+		
Triazines		+			+	
Phenylureas		+				+
Thioureas			+	+		

[a] Carrier stream: water-acetonitrile (50:50, v/v) containing 50 mM ammonium acetate (PI) or 25 mM tripropylammonium formate (pH 7.5) (NI). Flow rate, 1 ml/min. Discharge voltage, 1 kV. Adapted from [141]. LOD, amount corresponding to an ion trace of 500 counts multiplied by 10 (in order to compensate for additional peak broadening on changing from FIA/TSP–MS to LC/TSP–MS).

[b] Captan, fenaminosulf, sethoxydim, alloxydim and permethrin.

silvex in spiked water sample from Barcelona harbour [141]. Segmented-flow extraction into n-heptane was also applied for the LC/TSP–MS determination of organophosphorus insecticides, chlorophenols and phenoxyacetic acids; the organic phase was led through the sample loop of the injection valve of the LC system [143].

Ion-pair reversed-phase LC for the ionic compound difenzoquat and other quaternary ammonium pesticides was combined with post-column extraction (cf. [141,144]) and TSP–MS; a sulfonate-type counter ion allowed extraction of the analytes into the organic solvent. However, the high detection limits, typically 1–2 μg under full-scan conditions, indicate that the system cannot be used for environmental analysis without substantial improvement of the sensitivity.

Diquat and paraquat were studied by Yoshida et al. [145] who used reversed-phase LC with methanol/water containing 0.1 M ammonium acetate as eluent and SIM; detection limits were about 20 ng. It should be noted that quaternary ammonium compounds are not easily identified as such and that the above LC/TSP–MS methods are applicable for target compound analysis only. Unequivocal identification of quaternary ammonium compounds can be performed by liquid secondary ion mass spectrometry (SIMS), on the basis of negative ion spectra and adduct ion formation with matrix additives [146]. Such 'MS-only' methods, i.e. without LC separation, may be of help in environmental LC/MS analysis.

An interesting aspect is the variation in mass spectral information obtained when using TSP interfaces from different manufacturers. It is known [4] that e.g. the Hewlett-Packard interface has a higher tendency to form acetate adduct ions than the Finnigan MAT (4500) interface, which appears to be better suited for electron-capture processes [29,135,137, 142]. The use of filament off, filament on or discharge ionisation has only a slight influence on the abundance ratios of the quasi-molecular ions obtained from the different instruments.

LC/TSP–MS is often used complementary to GC/MS for the identification or characterisation of degradation products of various pesticides. Barceló et al. [147] studied the photodegradation of fenitrothion and propazine under different photochemical conditions in distilled water, artificial sea water and methanol/water solutions. GC/MS allowed the off-line characterisation of the various photolysis products of fenitrothion. The combined data from LC/UV diode-array detection and LC/TSP–MS permitted the identification of four photoalteration products of propazine in different types of water, using direct injection of the photodegradation solutions into the LC systems. The method is rather simple because there is no need for extraction prior to LC/MS characterisation of the breakdown products. The use of LC/TSP–MS however provides too little structural information, thus making identification difficult.

Betowski et al. [32] compared GC/MS and LC/TSP–MS/MS methods for ten analytes from the US EPA, SW-846 Method 8140, Organophosphorus Pesticide Parameters. Limits of detection for the analysis of soil samples using TSP ionisation were quite good and generally better than those attained by GC/MS. The degradation of naled to dichlorvos, observed with GC, did not occur with TSP introduction.

LC/TSP–MS was used in an interlaboratory study on the analysis of (nine) carbamate and phenylurea pesticides in the low mg/l range [148]. Results from nine participating laboratories showed an intralaboratory precision of LC/TSP–MS ranging from 6.5% to 33% relative standard deviation (RSD), whereas those from interlaboratory comparison

ranged from 30% to 98% RSD. The authors mentioned the day-to-day variations of TSP spectra as the most important parameter responsible for the unsatisfactory results. No real environmental samples were used in the study. A similar validation of TSP–MS and PB–MS methods is given in ref. [149].

Niessen et al. [33] explored the possibility to detect and identify minor constituents in benzothiazole-derived compounds by various MS techniques, such as GC/MS, and LC/MS with a moving belt, TSP or PB interface, and LC/TSP–MS/MS. A diode-array UV detector was used to trace the peaks. The results clearly demonstrated that more than one MS technique should be used in identification problems. The same group of workers investigated the possibility of gaining additional structural information under LC/TSP–MS and SFC/TSP–MS conditions [28]. Using the phenylurea diuron as an example, it was shown that the repeller voltage may sometimes be used to obtain structure-specific fragmentation, unfortunately at the cost of sensitivity. From the reported experiments it follows that the repeller voltage is a critical parameter in TSP–MS and that the optimum voltage is not the same for each analyte.

LC/FAB–MS and LC/TSP–MS techniques were used to identify compounds formed during in vitro metabolism of an experimental Monsanto chloroacetanilide herbicide [150]. Analysis with these techniques has several advantages since very little sample clean-up is needed and the individual analytes do not have to be isolated. Moreover, compounds such as acids can be analysed without derivatization. LC/FAB–MS yielded better results for the polar, and LC/TSP–MS for the less polar metabolites.

20.3.2 On-line and off-line trace enrichment

After initial studies, such as those discussed above, had shown the potential and limitations of LC/TSP–MS, it became clear that sample preconcentration and clean-up are necessary to reach the low detection limits required in environmental analysis. Interest expanded from studying MS processes to the total analytical approach.

Bellar and Budde explored the potential of off-line extraction and concentration techniques for the development of a broad-spectrum method for the determination of non-volatile target compounds in aqueous environmental samples [151]. They used liquid–liquid and liquid–solid extraction and subsequent gradient LC separation for samples spiked with carbamate, triazine, sulfonylurea, phenylurea and organophosphorus compounds. With the liquid–liquid preconcentration procedure, applied to pesticide levels of 2–50 μg/l, detection limits for 34 analytes varied from 0.2 μg/l for cyanazine to 18 μg/l for linuron in the filament-off, PI mode. Detection limits obtained via liquid–solid extraction, applied to pesticide levels of 20–500 μg/l, were approximately 10 times higher; the higher pesticide levels in the primary sample were required by the small size and limited capacity of the extraction cartridges used in this study. Combination of retention time data, molecular weight information from the $[M + H]^+$ and $[M + NH_4]^+$ ions, evidence from isotope patterns, and occasional fragment ions, provided satisfactory information for the identification of target analytes without a large risk of false positives. Using a similar method for the determination of 20 carbamate and phenylurea pesticides in crops, SIM detection limits in the PI mode were 0.25–1 mg/kg [152,153].

Off-line SPE was used for the concentration of 11 river water samples to 1 ml methanol extracts [40]. Injection of 20 μl aliquots enabled the detection of several triazine and

phenylurea pesticides at the 5 μg/l level with a double-focusing magnetic sector instrument, under full-scan conditions. A dichloromethane extract of a river water sample was used to provide confirmation of the identity of an alleged pollutant, isoproturon.

Volmer et al. [154] used off-line SPE and LC/TSP–MS, for the determination of pesticides in water samples. From a selection of 128 environmental pollutants, 95 compounds could be detected at the 0.1 μg/l level by using 1 l samples, gradient elution, post-column addition of the TSP buffer and PI time-scheduled SIM detection. The repeatability of the LC/TSP–MS procedure was better than 12% over a day. However, the long-term reproducibility generally was above 20%; besides, the long-term variation in TSP sensitivity remains a problem. In addition, an elegant method for the confirmation of analytes was presented, in which post-column addition is applied to change the TSP reagent conditions; thus, reactive ions with widely differing proton affinity were generated to induce or suppress molecular adduct ion formation.

Off-line trace enrichment on C-18-bonded silica or ion-exchange sorbents was used prior to LC/TSP–MS, for the analysis of atrazine and its metabolites in water [155]. LC/TSP–MS was applicable to both chlorotriazine and hydroxytriazine metabolites; the limits of detection were as good as those of conventional LC/UV diode-array detection (ca. 1 ng). The authors also studied supercritical fluid chromatography (SFC) with ESP detection, a large advantages of SFC/ESP–MS being the short time of analysis (<3 min) [108]. SFC/ESP–MS provided more than 10-fold better sensitivity (25 pg injected) than TSP, but only for the less polar chlorotriazine compounds.

A simple liquid–liquid extraction procedure has been applied prior to LC/TSP–MS to determine two sulfonylurea herbicides and a major metabolite of each of these in soil [156]. The method requires minimal sample preparation and enables the simultaneous analysis of four compounds down to the 20 μg/l level.

On-line trace enrichment prior to LC/TSP–MS was found to yield extremely low detection limits for phenylurea herbicides in surface and drinking water in a study of Bagheri et al. [157]. After trapping the analytes from a 50 ml sample on a precolumn, packed with a styrene-divinylbenzene copolymer or on membrane extraction disks, desorption was effected in the backflush mode with methanol/0.1 M aqueous ammonium acetate (40:60, v/v) and the analytes were transferred on-line to a C-18 analytical column for LC gradient separation. Using discharge ionisation PI detection, all phenylureas tested generated $[M + H]^+$ as the base peak, with $[M + NH_4]^+$ observed in some cases. In order to enhance selectivity and sensitivity, the chromatograms were recorded under time-scheduled SIM conditions. In river Rhine water samples spiked with a mixture of 15 phenylureas at levels ranging from 0.05 to 10 μg/l, detection limits for all compounds except linuron (60 ng/l) and chlorobromuron (120 ng/l) were found to be 5–15 ng/l. With this system, the presence of monuron and isoproturon at low ng/l levels in river Rhine water was confirmed.

The same system was used for the detection of 39 carbamate, triazine, phenylurea and organophosphorus pesticides [158]. Trace enrichment was carried out on precolumns packed with C-18-bonded silica. With 50 ml water samples detection limits for the solutes typically were in the 2–90 ng/l range using time-scheduled SIM. Low levels (0.005–2 μg/l) of simazine, atrazine, isoproturon and diuron were detected in three European rivers, Amsterdam drinking water and even in HPLC-grade water (Fig. 20.13, Table 20.2). A similar method was used by Chiron et al. [159] for the determination of 30 pesticides
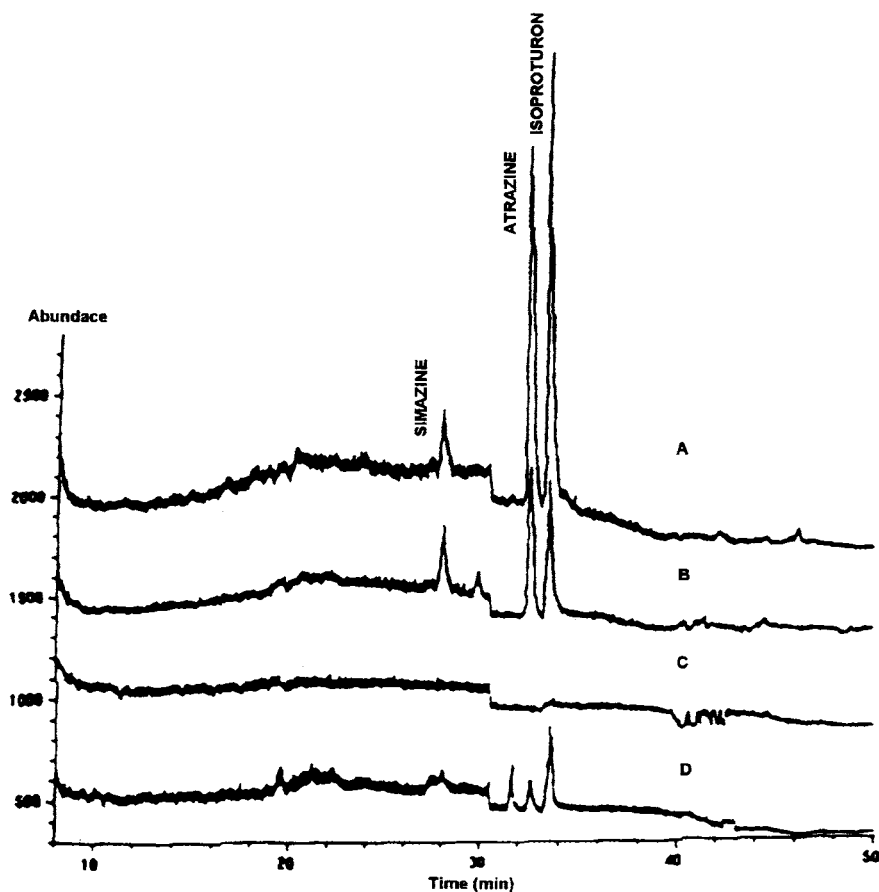
Fig. 20.13. On-line trace-enrichment-LC/TSP–MS trace for 200 ml of (A) River Rhine water, (B) Amsterdam drinking water, (C) blank without preconcentration and (D) HPLC-grade water. Column; 250 × 4.6 mm i.d. stainless-steel containing 5 μm C-18-bonded silica; eluent, linear methanol–0.1 M ammonium acetate gradient (10:90 to 90:10 (v/v) in 45 min); MS, discharge PI mode, time-scheduled SIM [158].

and various degradation products. Nine carbamates, triazines and anilides were found in surface and ground water samples at the 0.01–0.5 μg/l level. The automation of such systems has meanwhile been accomplished, although with a PB interface [63].

On-line SPE/LC/TSP–MS was also used in a 2-year monitoring study of a river basin [160]. The method has shown good sensitivity for a large number of compounds (typically 20–40 detected in a single sample), but the spectra did not provide much structural information. Nevertheless, it was possible to obtain molecular mass information and construct pollution profiles of the river basin. However, the compounds remained unidentified. RSDs of peak areas of internal standards added to each sample during the whole study were less than 7% ($n = 12$). The information provided by TSP–MS was found to be comparable to that of DAD UV and the latter detector was preferred in further studies.

Samples from the same river basin were analysed also by off-line SPE and LLE methods

TABLE 20.2

PESTICIDES DETECTED IN DRINKING AND SURFACE WATERS BY ON-LINE TRACE-ENRICH-MENT-LC/TSP–MS [158]

Water source[a]	Concentration (μg/l)			
	Simazine	Atrazine	Isoproturon	Diuron
Amsterdam drinking water	0.015	0.025	0.015	–
River Rhine	0.030	0.070	0.065	0.030
River Mersey	3.2	0.9	–	2.1
River Meuse	1.2	1.0	0.070	2.0
HPLC-grade water	–	<0.006	<0.007	–

[a] Sample volume, 200 ml.

followed by LC/TSP–MS [161]. Atrazine, desisopropylatrazine, monuron, isoproturon, metazachlor, 2,4-D and MCPA were determined in surface water at levels from 0.05 to 1 μg/l.

Similarly low detection limits, 0.02 μg/l, were obtained in the determination of the total content of the fungicides carbendazim, benomyl and thiophanate-methyl in water, using a column-switching LC/TSP–MS [162]. The latter two analytes were quantitatively converted into carbendazim and an aliquot of 10 ml of the reaction mixture was injected into the analytical system. Because the conversion makes the compounds indistinguishable, the detection limit is expressed as the carbendazim equivalent. The analyte detectability was poorer when the compounds were analysed by LC/PB–MS.

Both on-line and off-line SPE coupled with LC/TSP–MS were used for environmental monitoring of a series of nitrogen- and phosphorus-containing pesticides [163]. SIM detection limits for 51 selected compounds varied between 40–600 pg injected on-column which is equivalent to less than 100 ng/l in drinking water. In addition, structure - spectrum relations were investigated by means of APCI, ESP, FAB, ^{252}Cf plasma desorption and CID for several pesticides. Source and vaporiser temperature inducing thermal degradation, and post-column on-line derivatisation carried out by adding various alkylated amines to the LC eluent, were used to enhance the structure and molecular weight information from the TSP spectra.

One should note that the LC part of the system can be replaced by a simple FIA set-up, when neither analyte concentration nor separation is required. A fully-automated FIA/TSP–MS set-up [164] was used for structural confirmation of thousands of new agricultural chemicals. Switching of the mass analyser polarity between subsequent mass scans was utilised to obtain PI and NI TSP spectra for each injected compound within one chromatographic peak. Control of sample delivery, data acquisition and data output by a central computer was essential for consistency with Good Laboratory Practice; the approach might become a trend also in the routine use of other interfacing techniques.

SPE coupled on-line to LC/DAD UV followed by TSP–MS was used for degradation studies of 10 organophosphorus pesticides in estuarine waters [165]. In the experiment, 2-1 water samples were spiked at the 50 μg/l level with individual pesticides and exposed outdoor to ambient sunlight and temperature conditions; 100-ml sample aliquots were analysed on a weekly basis. TSP–MS in the PI mode was found useful for the confirmation

of numerous degradation products, e.g. fenthion sulfoxide, disulfoton sulfoxide, chlorpyr-iphos-oxon, pyridanfenthion and its oxon and fenamiphos sulfoxide. Assuming first-order degradation kinetics, the authors calculated the half-lives of tested analytes and three of their degradation products. On-line SPE/LC/DAD UV and GC/NPD were used for quan-tification.

An off-line concentration of eight fungicides, captan, captafol, carbendazim, chlorotha-nolil, ethirimol, folpet, metalaxyl and vinclozolin, from drinking, river and estuarine water was accomplished using C-18 membrane extraction disks [166] and LC/TSP–MS. Detec-tion limits were 0.5–2 μg/l when using TSP–MS operated in the SIM (PI and NI) mode. They were ca. 5–20-fold higher than those obtained with LC/DAD UV. Major interfer-ences found to be present in Empore disks by GC/MS were phthalates.

20.3.3 Tandem-MS and related techniques

Because of the generally rather poor fragmentation of TSP–MS, the coupling of a TSP interface with tandem-MS, with the goal of obtaining more relevant structural information, is of special interest [29,30,36–38].

Abián et al. [29] applied PI- and NI-mode detection in LC/TSP–MS and LC/TSP–MS/MS for the identification of atrazine, simazine, cyanazine, desethylatrazine, hydroxyatra-zine, and chlorodiamino-*s*-triazine. Detection limits in the SIM mode were below 400 pg; CID of $[M + H]^+$ ions, combined with product (daughter) ion scans or neutral loss scans of *m/z* 42 (C_3H_6) often provided higher detection limits (Table 20.3). It is noted here that MS/MS methods generally cause detection limits to become less favourable due to the ion intensity loss in the collision process. However, the intensity loss is often compensated for by the gain in selectivity and, thus, a better signal-to-noise ratio. It should be added that proper use of the technique requires optimisation of the collision energy, collision gas pressure, and quadrupole off-set correction parameter for each of the analytes studied. LC/TSP–MS/MS was applied to the characterisation of atrazine and its degradation products in aquatic photodegradation studies and in polluted soil samples [167].

More recently, the same authors investigated some ions of hitherto unknown origin, commonly observed in the TSP background spectrum [168]. Among other methods, TSP–MS/MS led to the identification of contaminants, e.g. acetamide, in the widely used ammonium acetate and formate eluent additives. These contaminants, which result from specific production processes, can be removed by washing the additive salt with chloro-form.

LC/TSP–MS/MS was used by Kienhuis for the screening of 20 pesticides [38]. Because of the difficulties encountered when using the MS/MS in the usual daughter, parent or neutral loss scan mode, a radio frequency-only daughter scan mode (RFD) was used in order to obtain more spectral information for the analytes studied. In the RFD scan mode the first quadrupole is operating as a high-pass mass filter, i.e., only ions with masses equal to or above the arbitrarily selected cut-off mass will enter the collision cell. When using the cut-off mass, low-mass signals due to CID of analytes become visible in a region of the mass spectrum, which would otherwise be dominated by the solvent. The third quadrupole acts as a mass analyser in the full-scan mode. By using two or three different alternating collision off-set voltages during one analysis, both molecular and daughter ions were acquired. By combining the diagnostic ions with different *m/z* values at both collision

TABLE 20.3

DETECTION LIMITS (ng) FOR TRIAZINE STANDARDS IN THE VARIOUS TSP–MS/MS ACQUISITION MODES (ADAPTED FROM [29])

Acquisition mode	Compounds studied[a]						
	CAAT	DIAT	HYAT	DEAT	CYAN	SIMAZ	ATRAZ
SIM[b]	0.4	0.4	2	0.4	0.4	0.4	0.4
DAU[c]	0.4	4	4	0.4	4	4	0.4
(SRM)[d]	(146–43)	(174–132)	(198–156)	(188–146)	(241–214)	(202–132)	(216–174)
NL[c] 42[e]	4	4	4	4	ND	ND	4
NL[c] 28[e]	ND	40	ND	ND	ND	40	ND
NL[c] 42 (SRM)[f]	4	4	0.4	0.4	ND	ND	0.4

[a] CAAT, chlorodiamino-s-triazine; DIAT, deisopropylatrazine; HYAT, hydroxyatrazine; DEAT, desethylatrazine; CYAN, cyanazine; SIMAZ, simazine; ATRAZ, atrazine. Detection limits (ng) calculated from the corresponding ion chromatograms. ND, not determined.

[b] Selected ion monitoring, alternate and continuous monitoring of the seven $[M + H]^+$ ions.

[c] DAU, daughter ion scan mode; NL, neutral loss scan mode.

[d] Transition for selected reaction monitoring, alternate and continuous monitoring of the seven indicated transitions.

[e] Acquisition in the scan range (Q1) 100–250 amu, 1 scan/s.

[f] Q1 focuses alternatively and continuously on the seven $[M + H]^+$ ions.

offset voltages, at least four ions, with an intensity of more than 10% of the base peak at each voltage, are available for 16 of the 20 compounds; no adduct ions were observed. Analyte detectability was at least as good as that obtained in the single-stage triple quadrupole MS scan mode. An off-line trace enrichment procedure with a carbon phase, followed by a LC separation, was used for the determination of ten pesticides. Using 500-ml spiked river Rhine water samples, 1 μg/l of each analyte could be detected.

Selectivity for characteristic ions of analytes is the main advantage of tandem-MS. In some cases direct-introduction MS/MS, without any separation, may be used for pesticide analysis. In the work of Chiu et al. [36], structure-specific fragmentation in the triple-stage quadrupole CID spectra of eight carbamates was used for the rapid screening of a carbamate mixture without the need for chromatographic separation (Fig. 20.14). Relevant data for all carbamates are tabulated in Table 20.4.

A method for the rapid screening of water samples for eight phenoxy-acetic acids and bentazone with FIA/TSP–MS/MS was reported by Geerdink et al. [37]. The analytes were introduced continuously into the system under NI conditions and two parent ion–product ion pairs were monitored for each analyte (one parent ion and three product ions for bentazone). 0.1 M ammonium acetate–acetonitrile (90:10, v/v) was found to be the optimum carrier stream. Without sample concentration all compounds could be detected at the 1 μg/l level, using time-scheduled selected reaction monitoring. Using a 5 ml loop injection, the total time of analysis was only 10 min. A similar strategy with off-line SPE for sample concentration was used by the same authors, where an optimised system [169] was applied for the analysis of 12 triazines and 11 triazine degradation products [170]. In recent work [171], the isolation of three triazines and nine triazine degradation products from surface water matrix was studied. The authors recommend a tandem-SPE procedure for sample clean-up; in the first step matrix compounds are removed by acidification of the sample to pH 1. Under these conditions the triazines are ionised and, consequently, not trapped on the C-18 SPE cartridge, whereas neutralised humic and fulvic acids are retained by the sorbent. The effluent pH is then re-adjusted to pH 7, the extract is concentrated and injected into the carrier stream for FIA–TSP–MS/MS. When using the tandem-SPE procedure, the quality of the monitored product–ion spectra improved considerably.

The dependence of the ion abundance in TSP mass spectra on the vaporiser and gas-phase temperatures under CID conditions has been studied for anilides, carbamates, *N*-heterocycles, organophosphorus and phenylurea compounds [30]. In the quoted study, 0.6 ml/min of a 150 mM buffer solution was added post-column to the LC eluent, which resulted in a final flow of 1.2 ml/min entering the TSP interface. A constant amount of added salt makes the limits of detection virtually independent of the gradient composition. A high water content was found to significantly improve the TSP sensitivity. For most of the analytes a linear relationship was found between the logarithm of the abundance ratio of the $[M + H]^+$ and $[M + NH_4]^+$ ions and the reciprocal of the absolute temperature of the gas-phase (range: 150–320°C). This may be of some value for SIM experiments because the total ion current (i.e., the sum of the $[M + H]^+$ and $[M + NH_4]^+$ ions) is less dependent on the gas-phase temperature than the ion currents of the individual quasi-molecular ions. Fragmentation is enhanced at higher salt concentration, but this disadvantage is outweighed by the strong increase in sensitivity, as was demonstrated for paraquat. For asulam, additional structural information was obtained by applying CID. No detection limits were reported.
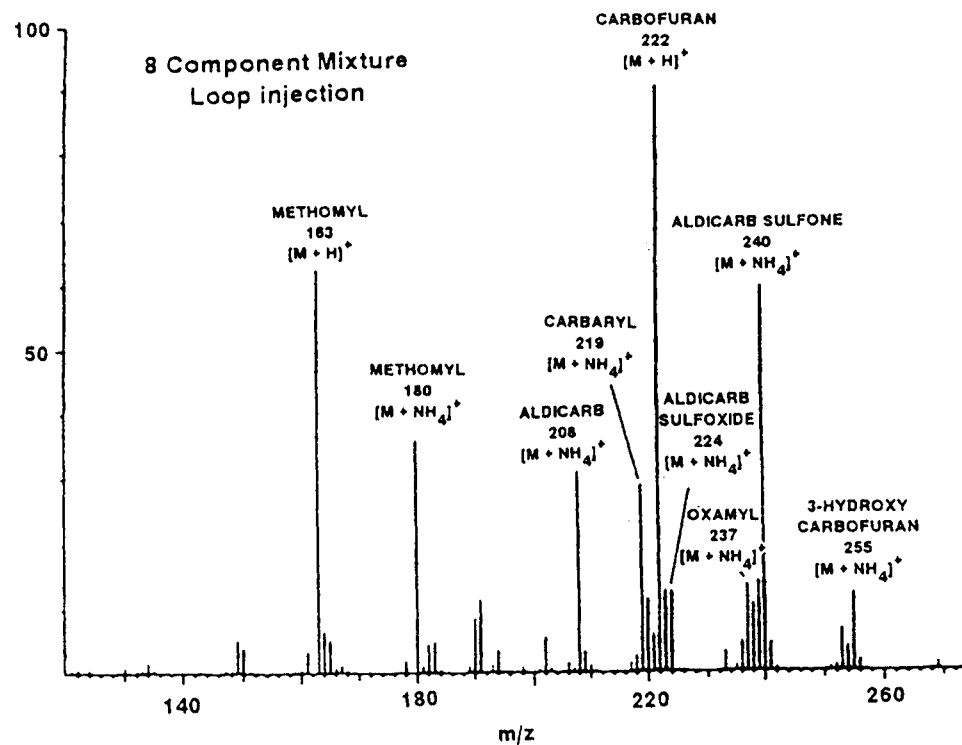
Fig. 20.14. Mass spectrum of the eight-component mixture obtained by FIA/TSP–MS/MS. Ions chosen for CID and related major product ions are reported in Table 20.4 [36].

TABLE 20.4

PRODUCT ION SPECTRA OBTAINED FROM EIGHT-COMPONENT CARBAMATE MIXTURE BY FIA/
TSP–MS/MS SCREENING ANALYSIS [36]

Compound	Peak	Ion chosen for induced dissociation (*m/z*)	Prodcut ion spectrum	
			m/z	Abundance (%)
Methomyl	$[M + H]^+$	163	106	100
			88	70
Carbaryl	$[M + H]^+$	202	145	100
			127	40
Aldicarb	$[M + NH_4]^+$	208	89	100
			116	30
Carbofuran	$[M + H]^+$	222	123	100
			165	30
Aldicarb sulfoxide	$[M + NH_4]^+$	224	89	100
			149	20
Oxamyl	$[M + NH_4]^+$	237	72	100
			90	20
Aldicarb sulfone	$[M + NH_4]^+$	240	166	100
			86	55
3-Hydroxycarbofuran	$[M + NH_4]^+$	255	163	100
			135	30

Draper et al. [31] studied phenols and the corresponding glucuronide and sulfate conjugates by LC/TSP–MS/MS, using NI detection. Nine model compounds were baseline-separated on a strong anion-exchange LC column using an aqueous ammonium formate–acetonitrile eluent. The use of a strong anion exchanger for the separation of the metabolite conjugates, proved compatible with TSP–MS. Under CID conditions the aryl sulfates fragmented efficiently to phenols, which were then detected as phenolate ions.

Coupling of a TSP interface to a magnetic sector MS [39] provides an interesting alternative to enhance the identification potential. With current data systems, an exact mass can be determined from a dynamic experiment where the analyte is introduced together with a reference; the exact mass of the analyte is obtained from interpolation with respect to the known reference masses. In preliminary experiments, ten carbamate pesticides were analysed in various fruits and vegetables; these compounds generally yielded a simple spectrum with $[M + H]^+$ or $[M + NH_4]^+$ as the base peak. Exact masses could also be obtained from a quadrupole MS, under special tuning conditions and using the data system capacities [43]. Although dynamic exact mass measurement under TSP conditions is not a routine procedure, neither with a magnetic sector nor with a quadrupole instrument, exact masses of molecular and fragment ions provide specific information for confirmation of analytes.

TSP–MS/MS was used by Minnaard et al. for the analysis of triazines in surface water [172]. In this study, the novel approach of single-short-column (SSC) for both sample enrichment and separation (for more details, cf. refs. [173,174] and Section 3.5) was used.

Analyses were performed in the PI filament-on mode, while triple-quadrupole MS/MS was used to record product ion spectra of protonated molecules. The triazines could be detected down to the 1 μg/l level by analysing 48-ml samples and their responses were linear in the range of 1–10 μg/l with RSDs better than 8%. The selectivity achieved by MS/MS was sufficient to identify atrazine in a mixture of 14 phenylurea herbicides (concentration, 50 μg/l each). The authors recommend the procedure for the rapid target analysis of a small number (4–6) compounds.

Photodegradation of alachlor and bentazone in distilled and river water under xenon arc lamp irradiation was studied by Chiron et al. [175]. The authors used both on-line SPE/LC/TSP–MS and on-line SPE/LC/TSP–MS/MS for the identification of degradation products. The use of the on-line technology provided better results than GC/MS and allowed to carry out experiments at low concentrations (20 μg/l), i.e., close to real-world situations. Next to additional confirmation by NMR, IR and GC/EI–MS, the authors also reported the synthesis of six transformation products of alachlor for final confirmation. In total, 14 photodegradation products of alachlor were found, while no significant breakdown products of bentazone could be identified. An example of gaining additional structural information for the identification of hydroxyalachlor by TSP–MS/MS is shown in Fig. 20.15.

Overviews of environmental LC/TSP–MS can be found in reviews by Lamoree et al. [4], Barceló [176] and Arpino [177].
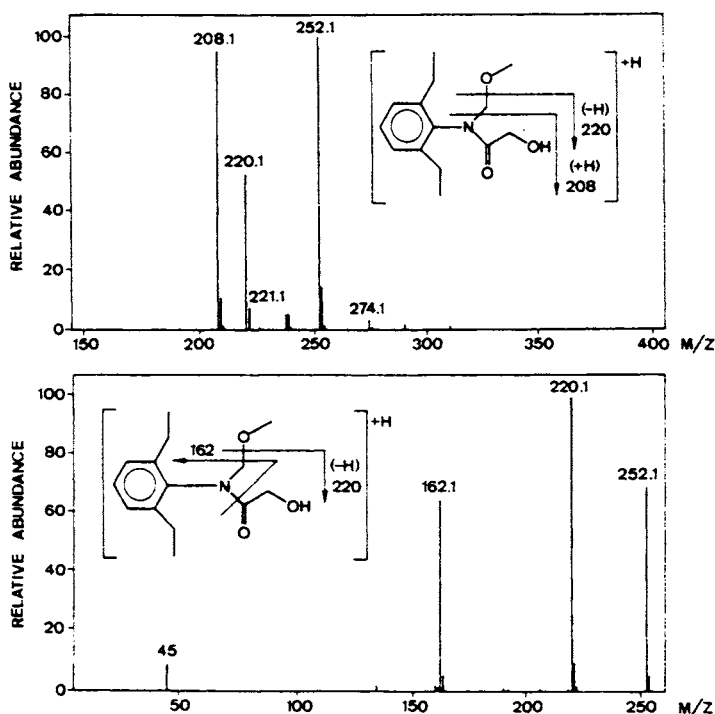
Fig. 20.15. LC/TSP–MS and LC/TSP–MS/MS spectra of hydroxyalachlor under positive ionisation mode [175].

20.3.4 Particle beam interface

Introduction of the PB interface gave mass spectroscopists the means to couple a wide range of LC separations to the ionisation technique of choice, especially in situations where identification of unknowns is required. Methods using the PB interface have successfully been applied to the analysis of a wide range of organic environmental pollutants since the appearance of the first systematic study on pesticides in 1990 [58]. In this pioneering work classical EI spectra were obtained for carbamates and phenylurea herbicides with instrument detection limits ranging from 10 to 440 ng in the full-scan mode. The potential of LC/PB–MS compared with LC/TSP–MS to obtain useful structural information is clearly demonstrated by the distinction of the isomeric triazine pesticides terbutylazine and propazine in ref. [178]. Unfortunately, the comparison of TSP and PB in terms of sensitivity was not addressed in that paper.

Miles studied the carbamate pesticide aldicarb and its degradation products, aldicarb sulfoxide and aldicarb sulfone, by a variety of methods, including GC/MS, LC/TSP–MS and LC/PB–MS [179]. LC/PB–MS yielded EI mass spectra with the characteristic fragment ion signals also observed in GC/MS. LC/PB–MS with CI detection did not provide more information than UV detection. The carbamate mesocarb was successfully screened with LC/PB–MS during the 1992 Olympic Games [180]. Its metabolite, a sulfate of p-hydroxymesocarb, could be detected in urine up to 48–72 h after the administration of a single 10 mg dose. This shows that some carbamates are amenable to LC/PB–MS, but that analyte detectability is not better than with other, more conventional LC detection methods.

Sulfate and glucuronide conjugates of substituted phenols were also studied by LC/PB–MS, using anion-exchange chromatography and EI or PCI detection [181]. Although the sulfates and glucuronides could not be identified as such, the phenol-type decomposition products were identified by their EI mass spectra. Instrument detection limits were between 0.25 and 51 ng for SIM detection under EI conditions.

LC/PB–MS of chlorinated phenoxyacetic acid derivatives allowed their identification, but showed that quantification was not reliable, because instrument response factors varied widely over a limited period of time [182,183]. Successful identification could be carried out by comparison with common library spectra and only small differences in the relative intensities were observed [182]. Evidence for thermal decomposition of chlorinated phenoxyacetic acid herbicides in LC/PB–MS was obtained by Betowski et al. [184]. Broadening of the profiles of some ions and variation in mass spectral quality were found to be dependent on the ion source temperature and analyte concentration; by implication, the instrument performance was found to depend upon the degree of fouling of the ion source. The authors suggested that PB mass spectra of chlorinated phenoxyacid herbicides are composite spectra of the parent herbicides and their thermal decomposition products.

The performance of TSP and PB interfaces was compared in an interlaboratory study [185]. NI-TSP–MS performed best in low-level (5 mg/l) detection, but PB with EI detection was a good means of identification at higher levels (500 mg/l). In view of the fact that low, 0.5–1 ng, levels of the same analytes could be detected by using a carrier (see below) in LC/PB–NCI–MS [186], it is likely that EI detection limits can also be improved.

More than 100 compounds from the US EPA National Pesticide Survey (NPS) were

used in a study on the feasibility of LC/PB–MS for the identification and quantification of residues of non-volatile pesticides in ground water [187]. Detection limits were estimated to range from 5 ng, for carboxim sulfoxide, to 50 ng, for disulfoton sulfoxide. The authors concluded that adequate analysis is only provided when results from LC/PB–MS are combined with results from other LC/MS methods, because LC/PB–MS is not sufficiently sensitive for the determination of all 104 NPS compounds studied. In a similar study on 40 NPS compounds [188], detection limits were found to be between 0.4 and 19.2 ng.

The compatibility of LC and PB–MS was extended by the application of on-line, post-column desalination of the eluent [189]. Reversed-phase/ion-exchange chromatography was performed using solutions of disodium carbonate and sodium hydroxide and chloride as the eluents, and desalination was achieved by cation exchange inside a microbore membrane suppresser, using a counter-current flow of sulfuric acid. Six aromatic sulfonic acids were separated and detected at the 0.4 μg/l level, using LC/PB–MS under full-scan EI conditions.

The above applications show that LC/PB–MS can be used successfully in pesticide analysis. However, the advantage of identification by PB–MS in the EI mode has to be set against the distinct disadvantage of rather poor analyte detectability and non-linearity of the detector response. Two main approaches have been developed to counteract these disadvantages: enhancement of sensitivity and linearity by the use of so-called carrier additives, and enhancement of sensitivity by off-line or on-line sample preconcentration. These aspects will be discussed below.

Various workers have used LC eluent additives to obtain improved detection limits. The effect of additives is generally twofold: the dependence of the detector response on analyte concentration becomes linear over a larger range and analyte detectability improves. Some studies on pesticide analysis have confirmed this 'carrier effect'. A possible interference due to a carrier effect was ruled out in the above mentioned study of analytes from the US EPA NPS [187]. In this work the linear or exponential response curves were considered to reflect a compound-specific mass transport efficiency with a distinct physical basis, precluding the addition of 'carrier compounds' to the eluent. A carrier effect was initially supposed to be most efficient if structurally similar additives were used. An ca. 2-fold intensity enhancement was observed for co-eluting isotopically labelled non-pesticide compounds [43]. No such enhancement was observed in the case of ethylenethiourea, a degradation product of the ethylene-bis-(dithiocarbamate) fungicides, and a ^{13}C isotopomer [190]. Full-scan detection limits of 5 μg/l were obtained for ethylenethiourea, when using the isotopomer as an internal standard. Chlorinated phenoxyacetic acid derivatives [186] and phenylureas [191] could be detected at low-μg/l levels upon the addition of a few μg/l of a structurally similar 'carrier compound' to the eluent. Moreover, the linearity of the concentration-to-response ratio improved. Methane NCI, combined with SIM, led to limits of detection of 1.1 μg/l for 2,4-D, 2,4,5-T and silvex [186], 0.16 μg/l for diuron and 0.5 μg/l for linuron [191]. The 'carrier effect' has also been observed for additives, which do not have any structural resemblance. In the initiating study mentioned above [58], ammonium acetate was added to the reversed-phase eluent in order to improve the transfer efficiency of the interface. With the analysis of the polar plant growth regulator damino-zide, using anion-exchange LC/PB–MS under isobutane PCI conditions, the presence of 0.4 mM maleic acid in the eluent resulted in a 30-fold signal enhancement, and gave a

linear response curve [57,192]. With 4mM maleic acid in the eluent, a detection limit of 25 μg/l could be obtained.

A discussion on problems of mass transport and calibration in LC/PB–MS is presented by Ho et al. [56]. These authors used 13 pesticides for the comparison of two PB interfaces in a study of the dependence of the transport efficiency on the design of the interface, the nature of the mobile phase, the vapour pressure and concentration of the analyte and the presence of coeluting carrier substances. In addition, 12 laboratories participated in an interlaboratory comparison of the analysis of four benzidines, in the 5–100 mg/l concentration range. RSDs of single analyst precision (<10%) and overall precision (<20%) were in the same order of magnitude as might be expected from GC/MS. External standards and a second-order regression curve gave satisfactory quantification results. However, on the basis of the observed enhancement of the signal by coeluting substances, a calibration method of coeluting isotopically labelled internal standards is suggested to be most reliable for real-world environmental samples. More details on isotope dilution in LC/PB–MS or GC/MS can be found in [55].

The use of LC/PB–MS for the analysis of effluent from waste water treatment plants was shown to complement GC/MS analysis [193]. Off-line sample preconcentration of 10-l waste water samples to 1 ml was followed by gradient LC, with the addition of 0.01% ammonium acetate as a carrier. The detection limit for triclocarban was found to be in the low-μg/l range. Obviously, identification of small amounts of non-target compounds by LC/PB–MS is feasible, but large samples must be available and preconcentration should be carried out.

In a study following the successful coupling of microflow LC and PB–MS [65], the performance of the set-up was tested with 45 selected pesticides (among others: carbamates, triazines, anilides, poly-chlorophenoxyacetic acids, organophosphorus and phenylurea compounds) [66]. Using off-line solid-phase extraction with graphitized carbon black material, pesticides were transferred from 2-l water samples to 100-μl aliquots. Detection limits, in the SIM mode, ranged from 1 to 40 ng of analyte injected with an injection volume of 60 nl; this corresponds to a concentration range of 0.2–30 μg/l in the original samples (at the assumption of 100% recovery). Reversed-phase LC was performed with laboratory-made capillary columns (C-18; 250 mm × 250 μm i.d.) and using 0.1 M ammonium acetate or trifluoroacetic acid as additives to the acetonitrile–water eluent for the separation of basic and neutral or acidic analytes, respectively. The authors reported a better response for high water content LC eluents during gradient runs (as compared to conventional-size PB) and linear calibration curves. A significant reduction in solvent consumption results in less contamination of the ion source and the pumping system [66].

As a further improvement of the micro-flow rate PB–MS, the authors covered the EI ion source with a PTFE layer, which has been proven to minimise analyte decomposition and adsorption [68]. Off-line SPE of 2-l water samples on graphitised carbon black (GCB) cartridges was used to determine 18 acidic pesticides, mainly (nitro- and chloro-) phenols and phenoxy acids. Phenoxy acids are heat-sensitive compounds, for which conventional PB–MS methods showed some limitations such as peak tailing in mass chromatogram due to their adsorption on the ion source surface and memory effects [65]. In order to improve handling at the trace level, higher injection volumes were used than in the previous work (500 nl vs. 60 nl). Instrument detection limits obtained in the FIA mode were 0.6–5 ng.

Assuming 100% recovery in the extraction procedure, the authors calculated SIM detection limits of all analytes in the range of 0.1–1 μg/l of surface water.

The micro-flow rate PB–MS system with a PTFE-modified ion source surface was used for analysis of 14 acidic pesticides [70] selected from the EPA method 515.1 for the determination of chlorinated acidic pesticides [194]. Using GCB extraction of 1-l spiked water samples, detection limits of LC/PB–MS operated in the SIM mode were from 0.7 to 7.2 ng/l (Table 20.5), which is ca. 10-fold better than with UV detection. Such detection limits are similar to those obtained with recent API–MS instruments.

Off-line trace enrichment methods are widely used, but on-line methods are gaining in popularity. On-line preconcentration using a precolumn with a PLRP-S sorbent, was applied for the analysis of water samples with LC/PB–MS [62]. Fig. 20.16 shows that four selected phenylurea compounds could be detected at the 30–50 ng/l level under full-scan EI conditions, using 100–250 ml samples. Calibration curves were linear in the 0.1–10 μg/l range and the day-to-day repeatability of LC peak areas was 3.6–9%. The same set-up was used for the identification of unknown pollutants in surface and drinking water; the results are summarised in Table 20.6. Low levels (50–250 ng/l) of the phenylurea compounds chlortoluron and diuron could be detected in surface water. A breakdown product of diuron, 3,4-dichloroaniline, could be positively identified by LC/PB–PCI–MS, using methane as the reagent gas.

Automation of the overall LC/MS set-up, including sample handling, seems to be an important step for the establishment as a routine methods for environmental analysis. A group of 48 carbamate pesticides and their degradation product were studied by means of automated on-line solid phase extraction/LC/PB–MS [63]; ionisation was performed by EI and ammonia and methane PCI or NCI. 100 ml surface water samples were enriched on a

TABLE 20.5

DETECTION LIMITS OF 14 CHLORINATED PESTICIDES IN WATER OBTAINED WITH LC/UV, GC/ECD AND LC/PB–MS IN SIM MODE; 1-l SAMPLES WERE CONCENTRATED ON GCB SPE CARTRIDGES [70]

Compound	HPLC/UV (ng/l)	GC/ECD (ng/l)	HPLC/PB–MS (ng/l)
Acifluoren	41.7	0.08	5.2
Bentazone	8.9	0.18	0.7
Chloramben	6.3	0.06	2.6
2,4-D	31.2	0.18	5.0
2,4-DB	83.3	0.19	0.7
Dicamba	9.6	0.07	4.5
3,5-Dichlorobenzoic acid	13.9	n.d.	0.7
Dichlorprop	20.8	0.17	1.5
Dinoseb	14.7	0.08	1.5
4-Nitrophenol	10.7	n.d.	1.5
Pentachlorophenol	12.5	0.05	0.7
Picrolam	8.3	0.12	7.2
2,4,5-T	30.6	0.83	4.5
2,4,5-TP	25.5	0.07	1.5

(10 × 3.0 mm i.d.) cartridge containing the sorbent of choice and, after switching of the eluent flow, the adsorbed pesticides were eluted by gradient LC onto a C-18 analytical column (Fig. 20.6). Due to efficient enrichment, it was possible to obtain detection limits of 0.1–8 μg/l in the EI full-scan mode for a group of 17 carbamates; relative standard deviations (peak areas) were between 5% and 20%. A systematic study of EI, PCI and NCI using both ammonia and methane reagent gases showed that ammonia PCI provided the best results of all CI modes used, while EI provided better sensitivity than PCI in most cases [195]. Fig. 20.17 shows some typical changes in PCI spectra of ethiofencarb with various reaction gases used. An increase by 2–3 orders of magnitude in TIC abundance was observed for polar degradation products as compared with their parent compounds; therefore, analyte detectability of the more polar degradation products is better. Surprisingly, 28 from the 48 carbamates could be satisfactorily detected by GC/MS; both the GC/MS and LC/PB–MS spectra could be identified by library searches (Fig. 20.18). Obviously, the two techniques are complementary for the analytes of interest.

A fundamental study comparing mass spectra of 14 carbamates obtained with desorption chemical ionisation (DCI) and FIA/PB–MS in ammonia-PCI mode was performed by Honing at al. [196]. The results indicate that thermal degradation takes place in the FIA/PB–MS system and well defined experimental conditions (ion source pressure and temperature) are of the utmost importance for the quantitative analysis.

An integrated, so-called MULTIANALYSIS system, was described by Slobodník et al. [197]. It combines LC and GC separations with a single mass spectrometer and SPE module. In the procedure, on-line SPE of 10–200 ml aqueous samples is followed by elution of the SPE precolumn in two subsequent runs, first to GC/MS and, next, to LC/PB–MS. Prior to entering the PB–MS, the LC eluent passes through the flow cell of a DAD UV detector (Fig. 20.19). The whole set-up was fully automated by means of a PROSPEKT, valve switching/solvent selection/cartridge exchange device, and controlled from the keyboard of the central computer. With such a system LC/PB–MS, LC/DAD UV and GC/MS data of the same sample were obtained within three hours. With 100-ml samples, detection limits of nine chlorinated pesticides (triazines, anilides and organophosphorus pesticides) in LC/PB–MS were 0.5–7 μg/l and in GC/MS 0.5–30 ng/l, both in the full-scan (EI) mode (Table 20.7); the RSDs of peak areas were 5–15%. The system, originally developed for water analysis, was used also for the analysis of sediments, using a so-called 'reconcentration-by-dilution' procedure in which the sediment extract is dissolved in 100–250 ml water and analysed as an aqueous sample. Several unknowns, amongst these a substituted phenanthrenone, were identified from their EI spectra and confirmed by NCI spectra.

The potential of NCI in the Multianalysis system was studied in a subsequent study [198]. From among three reagent gases, methane gave the best results for the above set of test analytes. For six pesticides, a 3–30-fold increase in sensitivity was observed compared with the EI mode and the dominant ionisation mechanism was electron capture (Table 20.7). Full-scan detection limits in LC/PB–MS were 50–200 ng/l.

The practicality of the Multianalysis system was tested during a 2-year monitoring study of the Nitra river basin (Slovak Republic) [160]. More than 30 compounds were identified by PB–MS in surface water and sediment samples at estimated levels 1–5 μg/l (Table 20.6). Results showed that the three techniques are complementary and should be used together for routine monitoring.

TABLE 20.6

SURVEY OF ENVIRONMENTAL CONTAMINANTS DETECTED IN VARIOUS WATER SAMPLES USING ON-LINE SPE/LC/PB–MS (ADAPTED FROM [62,160])

Compound[a]	Concentration (μg/l)	Water source	Ionisation mode
Triphenylphosphine oxide[b]	1	River Rhine	EI
	0.05	Amsterdam drinking water	EI
Chlortoluron[b]	0.25	River Rhine	EI
Diuron[b]	0.25	River Rhine	EI
	0.05	River Ebro	EI
	0.5	River Meuse	EI
	7	Effluent water	EI, NCI
Bis(2-hydroxyphenyl)methane[b]	0.1	River Ebro	EI
Bis(4-hydroxyphenyl)methane[b]	0.1	River Ebro	EI
N-Butylbenzenesulfonamide		River Ob	EI
Tris(2-chloroethyl) phosphate		River Meuse	EI
3,4-Dichloroaniline[b]		River Meuse	EI, PCI
N-(3,4-dichlorophenyl)-N′-methyl urea		River Meuse	EI, PCI, NCI
Bentazone[b]	70	Effluent water	EI, NCI
2,4-Dichlorobenzoic acid[b]		Effluent water	EI, NCI
2(1H)-Quinolinone		Sewage sludge	EI
2,3-Dihydro-4-methyl-1H-indole		Sewage sludge	EI
1,4,7,10,13,16-Hexaoxacyclo-octadecane		River Nitra	EI
Ortho-tolylbiguanide		River Nitra	EI
N-[2-(2-Oxopropyl)phenyl] acetamide		River Nitra	EI
Tetraisopropylidene-cyclobutane		River Nitra	EI
Trimethoprim		River Nitra	EI
1,1′-(2,2-Indolizinediyl)bisethanone		River Nitra	EI
N-2-Methyloctadecanoyl pyrrolidine		River Nitra	EI
2-(Isopropylamino)-1,4-naphthaquinone		River Nitra	EI
3-Hydroxy-dimethyl-pentanedionic acid		River Nitra	EI
2-(3-Thienyl)ethyl-1-(2-dibenzo-thienyl)		River Nitra	EI
2,5-Didesoxy-tri-o-(trimethylsilyl)-pentitol		River Nitra	EI
4,4′-(1-Methylidene)bisphenol		River Nitra	EI
1-(2-Methoxy-1-methylethoxy)-2-propanol[c]		River Nitra	EI
6-Deoxy-2,3,4,5-tetrakis-D-galactose		River Nitra	EI
4-Ethylpyridine		River Nitra	EI
Atrazine[c]		River Nitra	EI
3-Phenyl-4-methylisoxazol-5-one		River Nitra	EI
2-(Tetradecyloxy)ethanol		River Nitra	EI
Tetradecene[c]		River Nitra	EI

TABLE 20.6 (*continued*)

Compound[a]	Concentration (μg/l)	Water source	Ionisation mode
Bis(2-ethyl)-1,2-benzene-dicarboxylic acid		River Nitra	EI
Tetrabutylammonium		River Nitra	EI
(1-Propyloctyl)benzene		River Nitra	EI
2-Anthracenamine		River Nitra	EI
2-Propenoic acid, 3-(4-methoxy-phenyl), 2-ethylhexyl ester		River Nitra	EI
Octadecanoic acid, 3-methoxy-, methyl ester		River Nitra	EI
Monopentyl-1,1-benzene-dicarboxylic acid		River Nitra	EI
Tetraethyl plumbane		River Nitra	EI
2,3,4,4a,10,10a-Hexahydro-6-hydroxy-1,1,4a,trimethyl-7-(1-methylethyl)-,(4a*S-trans*)-,9(1*H*)-phenanthrenone[c]		River Nitra sediment	EI

[a] Non-IUPAC names were taken directly from the spectrum library.
[b] Checked by injection of standard.
[c] Confirmed by GC/MS.

A combination of PB–MS EI and PCI spectra was used in degradation studies of eight pesticides, isoproturon, alachlor, aldicarb, diuron, atrazine, fenitrothion, methiocarb and metoxuron [199]. Analytes were spiked at a level of 50 μg/l into a vessel with surface water, which was placed under the medium-pressure mercury lamp. 50-ml aliquots were taken at regular intervals and analysed by SPE/LC/DAD UV/PB–MS, automated by means of an OSP-2A valve switching, solvent selection, cartridge exchange device. Each experiment was performed twice, once with PB–MS operated in the EI and, then, in the PCI ammonia mode. The results were compared with direct loop injections of 25-μl aliquots from samples spiked at high (100–200 mg/l) levels. Relevant example is shown in Fig. 20.20. Ions of protonated and ammoniated molecules in the PCI mass spectra were used to determine the molecular masses, and EI mass spectra for unambiguous identification and/or structural confirmation. Additional information was obtained from the UV DAD detector placed between the outlet of the analytical column and the MS detector. More than 40 photodegradation products of pesticides were detected and, in many instances, tentatively identified.

On-line SPE/LC/DAD UV/PB–MS was also used by Marcé et al. [200] for the determination of carbamates, triazines and phenylureas. Detection limits for 100-ml samples were 0.02–0.5 μg/l for PB–MS and 0.05–0.5 μg/l for DAD UV detection. The authors observed a distinct improvement of PB–MS responses due to coeluting compounds from the matrix, and recommend the use of standard addition for quantification. Isoproturon and *N*-benzenesulfonamide were identified at sub-μg/l levels during the analysis of real-world samples. Similar on-line approach was used for analysis of triazines in drinking and surface water whereas off-line SPE was used for analysis of soil samples [201].
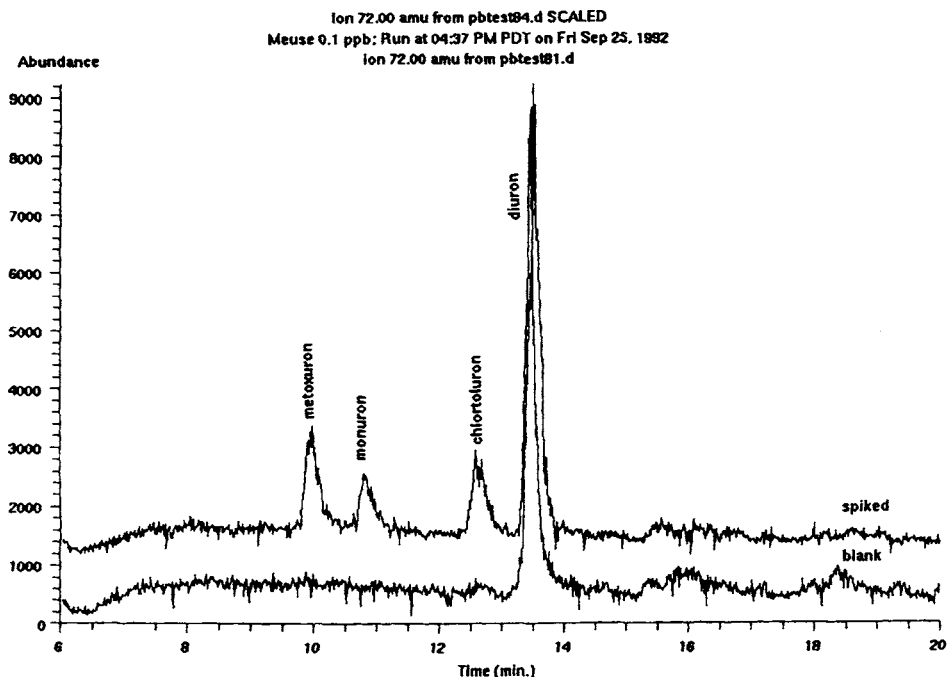
Fig. 20.16. On-line trace-enrichment-LC/PB–MS chromatogram of 250 ml of (bottom) River Meuse water (blank) and (top) River Meuse water spiked with 0.1 μg/l of a mixture of four phenylureas. Column: 250 × 4.6 mm i.d. stainless-steel containing 5 μm C-18-bonded silica; eluent: linear acetonitrile–0.1 M ammonium acetate gradient (50:50 to 95:5 (v/v) in 30 min); MS, full-scan EI mode. In the blank sample diuron was detected [62].

In a new approach for the rapid analysis of organic micropollutants in aqueous samples a so-called single-short-column (SSC) was used for both trace enrichment and separation; detection was performed by UV DAD and PB–MS [202]. Detection limits of four (from six) phenylureas were 1 μg/l and 0.1 μg/l in surface water, when the PB–MS was operated in the full-scan and SIM mode, respectively. With this set-up, the total time of analysis could be reduced to 32 min. Promising results stimulated further research and the SSC was later also coupled to TSP–MS and API–MS [97,172–174].

LC with a PB interface was successfully coupled to an ITMS [60]. The performance of the PB–ITMS set-up was tested with, amongst others, caffeine and carbaryl. Apart from the usual problems, such as relatively low sensitivity, non-linearity and analyte condensation on the first skimmer, ion–molecule reactions were found to occur at high analyte concentrations. Preliminary results show that instrument detection limits with PB–ITMS are in the low-ng range (high-μg/l range with the applied injection volumes).

The above examples show that the instrument detection limits in PB–MS generally are rather high. This is a serious disadvantage in environmental analysis. There is no doubt that the possibility to obtain reproducible EI and solvent-independent CI spectra is the main driving force for further development of PB; in this respect it is superior to all other LC/MS interfaces presently available. Improving the analyte detectability by adding

Ethiofencarb (MW 225)
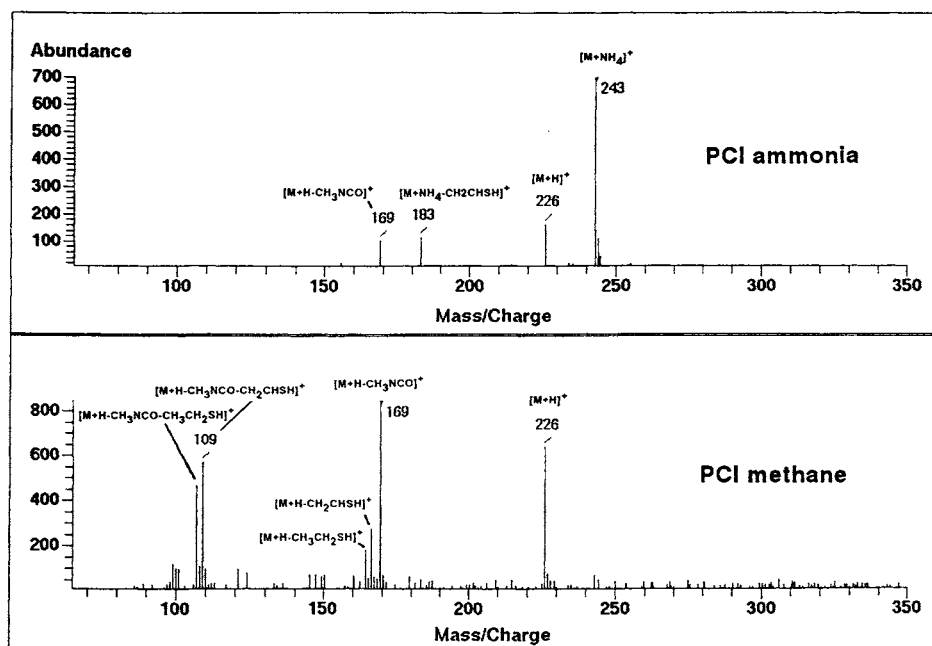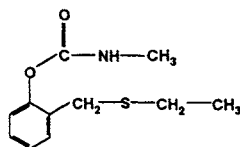
Fig. 20.17. FIA/PB–MS spectra of ethiofencarb (500 ng) in PCI mode using ammonia and methane as reagent gases [63].

specific additives does not seem to apply universally and attention is nowadays rather devoted to efficient trace enrichment prior to LC separation. The efforts led to the incorporation of LC/PB–MS into a standard US EPA procedure for the determination of benzidines and nitrogen-containing pesticides in drinking water [203].

The performance of LC/PB–MS and LC/APCI–MS for the analysis of 15 triazines, phenylureas, thiocarbamates, chlorophenoxy acids, organophosphorus and phenolic compounds was compared in [204]. PB–MS was operated in the EI or methane PCI/NCI modes. APCI–MS data were obtained in both the PI and NI modes. The ions of protonated $[M + H]^+$ and deprotonated $[M - H]^-$ molecules were the base peaks in APCI–MS. Similarly in PB–MS, $[M + H]^+$ ions in PCI and $[M - H]^-$ ions in NCI mode were obtained as major peaks. In both methods, an on-line C-18 SPE of 200-ml samples was used prior to the LC separation. Using time-scheduled SIM, detection limits of PB–MS were 50–200 ng/l and those of APCI–MS, 0.8–20 ng/l (Table 20.8). Atrazine and simazine were detected by APCI–MS in an Ebro river sample at a level of ca. 0.05
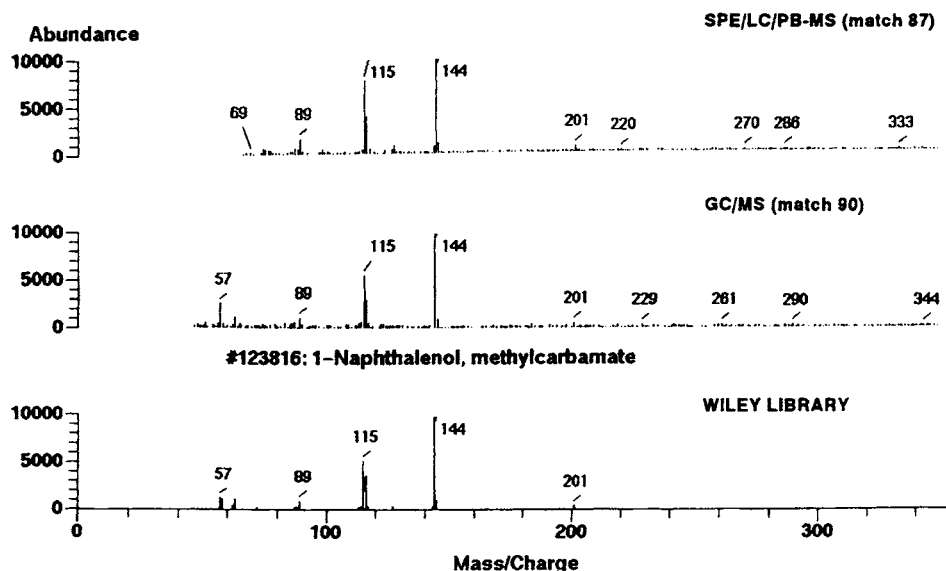
Fig. 20.18. EI spectra of carbaryl (MW 201) obtained after on-line trace-enrichment-LC/PB–MS of 1µg/l of analyte in 100 ml surface water, GC/MS of 2 ng analyte injected on-column, and Wiley/ NBS spectral library search. Values in brackets indicate the quality of spectral match in comparison with standard reference spectrum in Wiley/NBS library (maximum 100) [63].

ng/l, while simazine did not show up in the PB–MS chromatograms. The PB–MS chemical ionisation and APCI–MS spectra were rather similar. Both methods were satisfactorily validated in the Aquacheck interlaboratory study [205].

An on-line dual-precolumn SPE set-up was coupled to LC/DAD UV/PB–MS by Hogenboom et al. [173] to analyse 28 micropollutants, e.g. neutral pesticides, phenolic compounds and acidic herbicides, which covered a wide polarity range. The analytes were selected from a wider group of 110 analytes of environmental interest. In a typical analysis, a 40-ml sample was led through the 'first' precolumn and the last 10 ml through both precolumns. The 'second' precolumn then contains highly polar compounds with low breakthrough volumes (but no interfering, early eluting humic and fulvic acids) and is eluted first. Next, the 'first' precolumn is desorbed onto the analytical column, all within the same run. Even with the small sample volume (10 ml) used detection of, e.g. phenol and m-cresol in surface water is possible at 0.1 µg/l. The whole set-up was fully automated as a part of the SAMOS system [61]. Another selection of the analytical column was found to be a critical parameter during optimisation of the whole procedure; the use of a column packed with 3.5-µm particle size Zorbax-SB C18 was recommended. With the above approach it was possible to detect simultaneously compounds as widely different as, e.g., phenol, pentachlorophenol, bentazone and isomeric dichlorobenzoic acids. The latter two compounds were identified on the basis of their EI mass spectra and then confirmed by NCI using ammonia as a reagent gas. Among other identified compounds in real-world water sample were 2,4-dichlorobenzoic acid, carbamazepine, diuron and tris(2-butoxyethyl)phosphate.
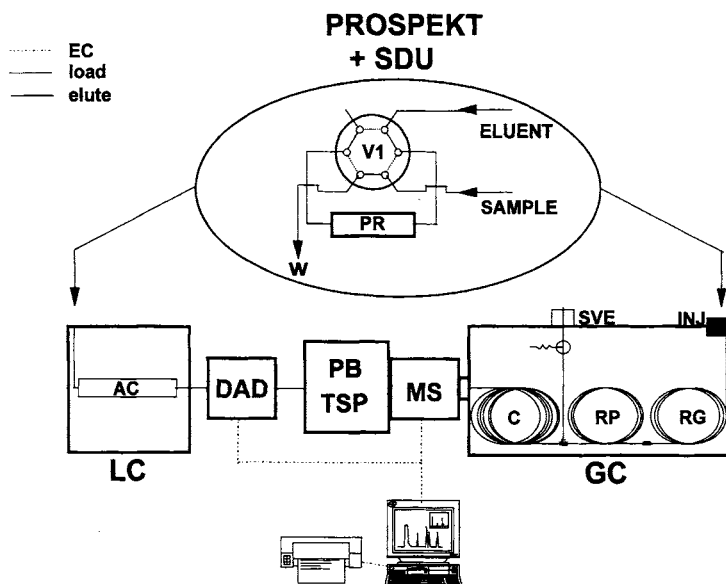
Fig. 20.19. Simplified scheme of the on-line SPE/LC/MS set-up using PB and GC/MS [160]. ELUENT, LC eluent gradient or ethyl acetate for GC; EC, electronic connections; V1, six-port switching valve; load/elute, positions of V1; PR, precolumn or cartridge; W, waste; AC, analytical column; LC, liquid chromatograph; DAD UV, diode-array detector; PB, particle beam interface; TSP, thermospray interface; MS, mass spectrometer; GC, gas chromatograph; C, GC analytical column; RP, retaining precolumn; RG, retention gap; SVE, solvent vapour exit; INJ, on-column injector.

20.3.5 Atmospheric pressure ionisation

The rapid development of API–MS has attracted much attention from environmental chemists. In our previous review, from 1995 [6], we stated that 'very few studies on LC/API–MS for the determination of pesticides have been published so far, despite the potential of API–MS techniques in terms of sensitivity and the provision of structural information which is often claimed'. Today, this statement is not valid anymore: the majority of new LC/MS applications for the determination of pesticides utilises API–MS technology.

A mixture of selected carbamates and phenylureas was analysed by means of simultaneous ISP–MS and APCI–MS, using gradient LC with ammonium formate as an additive [87]. The double detection was achieved by splitting the effluent of the analytical column (0.4 ml/min) to deliver ca. 20 μl/min to the APCI interface. A potential of 20–40 V over the ion sampling capillary and the first skimmer was used to effect solvent cluster breaking and induce in-source CID; this resulted in an improved signal-to-noise ratio. The observed difference in the TIC responses from ISP and APCI was attributed to thermolability of the analytes and to differences in the ionisation mechanism. The CID–ISP and CID–APCI mass spectra displayed several characteristic fragment ions. Unfortunately, no detection limits or repeatability data were reported.

TABLE 20.7

DETECTION LIMITS OF ON-LINE SPE/GC/MS AND SPE/LC/PB–MS FOR 100-ml TAP WATER SAMPLES [198]

Analyte	SPE/GC/MS (ng/l)		SPE/LC/PB–MS (μg/l)	
	EI	NCI	EI	NCI
Atrazine	1	100	0.5	>10
Alachlor	7	–	7	–
Fenchlorphos	5	0.2	5	0.2
Cyanazine	10	3	0.5	0.15
Metolachlor	0.5	–	0.5	–
Chlorpyriphos	30	1	5	0.2
Bromophos	5	0.3	3	0.2
Tetrachlorvinphos	1	0.1	0.5	0.15
Coumaphos	30	2	0.7	0.05

The performance of two API-based (ISP and APCI) LC/MS procedures and of the more established TSP and PB methods was compared in a detailed study on *N*-methylcarbamates [206]. It is shown on example of methomyl that ISP, APCI and PB have the advantage of identification over TSP. As regards the sensitivity of detection, APCI showed ca. 10-fold better detection limits than ISP and TSP. Results obtained with PB–MS were the least satisfactory: in some instances this method was almost four orders of magnitude less sensitive than APCI–MS. However, such poor PB–MS performance does not agree with results of other authors [63]. Carbaryl could easily be detected in green pepper extracts at the 0.1 mg/kg level by ISP–MS and ISP–MS/MS. At this level, confirmatory full-scan spectra of methomyl, aldicarb and carbaryl could be obtained with LC/APCI–MS.

Conventional liquid–liquid extraction of water samples with dichloromethane was used prior to LC/APCI–MS for the determination of 12 triazines, phenylureas, acetanilides and organophosphorus pesticides [207]. Using 1-l ground water samples, SIM detection limits obtained by monitoring the ions of protonated molecules were 1–6 ng/l, which corresponds to ca. 50–300 pg injected on column. Over 200 samples were analysed during a 1-year national survey of ground water pollution in Denmark; the method was robust. Metamitron, simazine, atrazine, isoproturon, terbuthylazine and the degradation products desethylatrazine and desisopropylatrazine were found in real-world samples at concentrations from 1 ng/l up to 19 μg/l.

LC/ISP–MS was used for the determination of nine organophosphorus pesticides [81]. Off-line trace enrichment of 500-ml water samples on various Empore disks or C-18 cartridges was used for enrichment of the analytes. Limits of detection from direct on-column injections were 10–200 pg. With 1 ng of each compound injected, the repeatability varied from 12% to 17% and the long-term reproducibility was 22–30%. It was possible to detect 0.1 μg/l of each analyte in the SIM mode using the $[M + Na]^+$ adduct ions. Unfortunately, no full-scan detection limits were reported. The authors observed that the ISP spectra display diagnostic ions of organophosphorus pesticides which could be used for the identification of unknowns. Comparison of LC/ISP–MS and LC/TSP–MS of
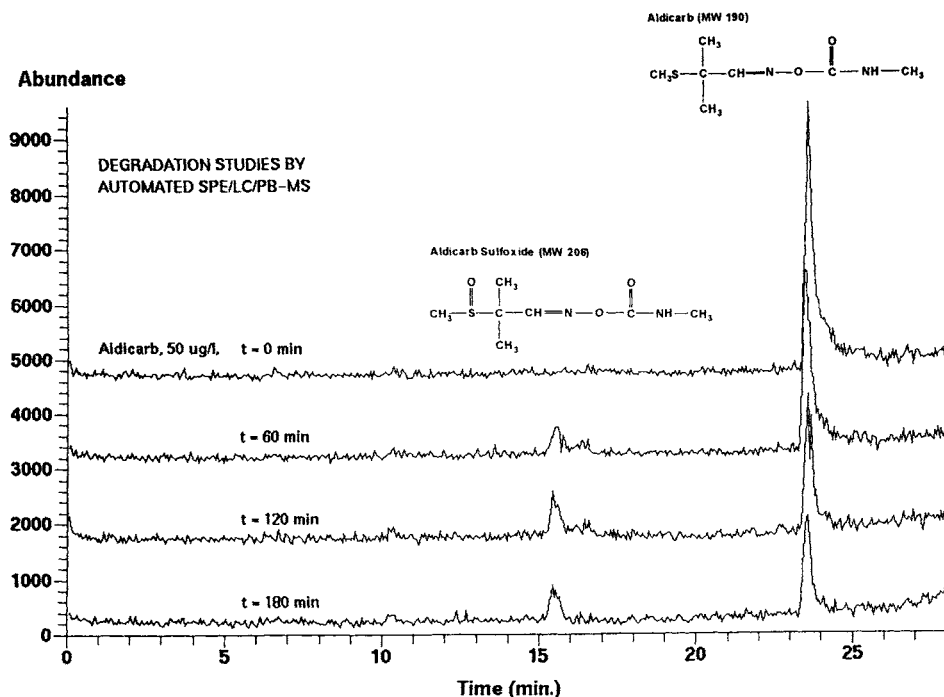
Fig. 20.20. Extracted ion chromatograms (m/z 68) obtained by SPE/LC/PB–MS of 50-ml river Rhine water samples spiked with 50 μg/l aldicarb. Sample was kept under a medium-pressure mercury lamp; sample aliquots were collected and analysed in 60-min intervals. Aldicarb sulfoxide was detected as a degradation product of aldicarb [199].

the same compounds, showed that ISP is to be preferred: it has about 100-fold better sensitivity, and thermal degradation of trichlorfon, observed for TSP, was not found with ISP.

TABLE 20.8

SIM DETECTION LIMITS OF EIGHT PESTICIDES (IN μg/l) OBTAINED BY ON-LINE SPE/APCI–MS AND SPE/LC/PB–MS OF 200-ml TAP WATER SAMPLES [204]

Compound	SPE/LC/PB–MS			SPE/LC/APCI–MS	
	EI	PCI	NCI	PI	NI
Ametryn	0.05	n.d.	0.1	0.001	n.d.
Atrazine	0.05	0.02	n.d.	0.0008	n.d.
Isoproturon	0.05	0.02	n.d.	0.002	n.d.
Malathion	0.1	n.d.	0.02	0.004	0.01
Fenitrothion	0.2	0.1	0.05	n.d.	0.01
Parathion-ethyl	0.2	0.1	0.05	n.d.	0.005
Prometryn	0.05	0.02	0.05	0.0008	n.d.
Terbutryn	0.05	0.02	0.05	0.0008	n.d.

Seventeen pesticides from the US EPA NPS of ground water contaminants, amongst others triazines, carbamates, phenylureas and organophosphorus compounds, were analysed by LC/APCI–MS [93,208]. Detection limits were 0.8–10 ng under full-scan conditions, and 0.01–1 ng under SIM conditions. The full-scan APCI–MS detection was found to be less sensitive to differences in analyte structure (range, 10-fold) than TSP–MS (50-fold) or PB–MS (150-fold), at least for the present pesticides. Compared with the above mentioned study, the detection limits of four (from eight) analytes in APCI–MS were about the same as in TSP–MS, and PB–MS showed satisfactory performance. The in-source CID-APCI mass spectra of aldicarb sulfone and carbaryl provided useful structural information and were found to be virtually identical to the triple-quadrupole MS/MS CID mass spectra.

Conboy at al. [209] used a combination of ion chromatography coupled on-line with ion-pairing agent removal technology, and ISP–MS. The LC column effluent was led into a cation suppresser, between two ion-exchange membranes which were continuously regenerated by a counterflow of aqueous acid. The suppresser dead volume was less than 50 µl, to avoid peak broadening. After cation suppression, the 0.8–1.0 ml/min LC flow was split, such that 10–20 µl/min could be directed to an ISP–MS interface. This method was used to study quaternary ammonium compounds, using MS and MS/MS for detection. The limit of detection of 40 pg tetrapropylammonium, injected on-column, is an order of magnitude better than that obtained with a conductivity detector. For the identification of some alkyl sulfates and sulfonates, using NI detection, no limits of detection are given. The fact that the method is applicable to polar, ionic and zwitterionic compounds, justifies further research for environmental analysis.

The introduction of a high-flow LC/ISP–MS system [88] caused a breakthrough in this area. Conventional LC flow rates of 1–2 ml/min were used in ISP with a heated ion sampling capillary and a liquid shield. This system was reported to provide mass spectra of low-ng quantities of compounds injected on-column. Non-volatile phosphate buffers could be used in the LC eluent without major problems. 100 ng/l of mexacarbate could be detected in spiked pond water, after SPE of a 150-ml sample over a C-18 cartridge; the entire extract, which contained 3 ng of the analyte, was injected on-column. Mexacarbate, monuron, propoxur and siduron were determined in the SIM mode, using 10–25 ng on-column injections. Carbamates could be detected at low-ng levels, using gradient LC for separation. The high-flow ISP system parameters have been studied later by the same group, using alkylbenzoate esters, monuron and carbofuran as model compounds [86].

LC/APCI–MS and LC/ESP–MS were used in a 2-year survey of a river basin [161]. Atrazine, simazine, desethylatrazine, malathion, fenthion, cycluron and several phthalates were identified with the two techniques. However, not unexpectedly, for the relatively apolar phthalates APCI–MS provided better sensitivity than ESP–MS. Despite the combined use of numerous techniques, including GC/MS and GC/AED, the majority of the detected compounds could not be identified.

ISP–MS using a narrow-bore LC system was used for the multiresidue determination of carbofuran, promecarb, linuron, monolinuron, atrazine and its degradation product hydroxyatrazine in drinking water [210]. To meet the requirements of the maximum admissible concentration in drinking water, analyte enrichment was performed using off-line C-18 SPE. The authors used an elegant method of simultaneous collection of ions of (i) protonated $[M + H]^+$ and sodiated $[M + Na]^+$ molecules and (ii) characteristic fragment ions,

obtained at low (20 V) and high (60 V) in-source CID voltages. Repetitive jumps between the two voltages were produced every second. Characteristic results for atrazine and promecarb are shown in Fig. 20.21. Similar results and ion types were reported previously [211] using tandem MS. The ISP–MS operated in the SIM mode provided detection limits of 7–25 pg, which allowed determination of all pesticides but hydroxyatrazine at 0.04–0.08 μg/l in water samples. Detection limits in the full-scan mode were ca. 100-fold higher.

LC/ESP–MS was used for the determination of analysis of acetochlor, alachlor and metolachlor and their chloroacetanilide metabolites–oxanilic and sulfonic acids [212]. 100-ml ground and surface water samples were enriched off-line on C-18 cartridges and selectively eluted in two steps; first with ethyl acetate (parent compounds) and then with methanol (metabolites). Using ESP–MS in the NI mode, SIM detection limits for the metabolites were 0.01 μg/l, which represents ca. 200 pg injected on-column. All compounds gave highest responses at a CID voltage of 20 V, showing an abundant molecular ion or the ion of deprotonated molecule. Increasing the CID voltage to 60 V caused additional fragmentation of the sulfonic acid metabolites - however, at the price of a significant decrease in sensitivity, which would make these conditions unsuitable for routine analysis. The coefficients of variation were rather high, viz. 25–35% and 15–20% for surface water and groundwater samples, respectively, which was due to plugging problems in the ESP source and variations in the solvent flow-rates.

A multiresidue LC/ESP–MS method was developed by D'Ascenzio et al. [213] for the low- and sub-ng/l level determination of 15 sulfonylureas, imidazolines and arylphenoxypropionic acids in drinking and groundwater samples. They used off-line SPE 4 l drinking or 2 l ground water on GCB sorbent. Detection limits were 3.8–9.9 ng/l (full-scan) and 0.5–4.5 ng/l (TS-SIM). The method was linear from 5 to 250 ng for each analyte. The authors conducted a study on the suppression of 'unwanted' sodium/potassium adduct ion formation by adding formic acid to the reconstituted samples, the optimum concentration being 10 mmol/l. The results were in good agreement with independent studies by Zhou [214] and Pleasance [206]. The capacity of the GCB cartridges to extract acid analytes from water without any sample pretreatment (recoveries >87%) was found a substantial advantage compared with C-18 material (recoveries, 18–82%). Five analytes were determined at sub-100 ng/l levels in a real world survey of groundwater samples from three different locations in Italy.

The excellent sensitivity of ESP–MS and ESP–MS/MS was utilised for the direct analysis of six imidazolines in water samples at the 1 μg/l level without any sample concentration step [215]. After filtration, 10-μl samples were directly injected onto a 5 cm × 4.6 mm i.d. LC column packed with 2 μm C-18-bonded silica. Enhanced chromatographic performance resulted in narrow (<4 s) peak widths at half height and up to 8-fold improved signal-to-noise ratios compared with usual (10 cm × 3.0 mm i.d.) C-18 column. The total analysis time was 10 min. Recoveries of all analytes were 96–105% (RSDs, 2.6–7.5%) and the calibration curves were linear from 0.5 to 10 μg/l. CID of protonated molecules in the triple-quadrupole instrument generated a number of structurally significant product ions, especially ions m/z 86 and 69, common to all imidazolinones. When monitoring the ion of the protonated molecule (first quadrupole) and m/z 86 (third quadrupole), responses in LC/ESP–MS/MS were ca. 10 times lower than those obtained with LC/ESP–MS ([M + H]$^+$). This was attributed to losses during ion transmission in the MS/
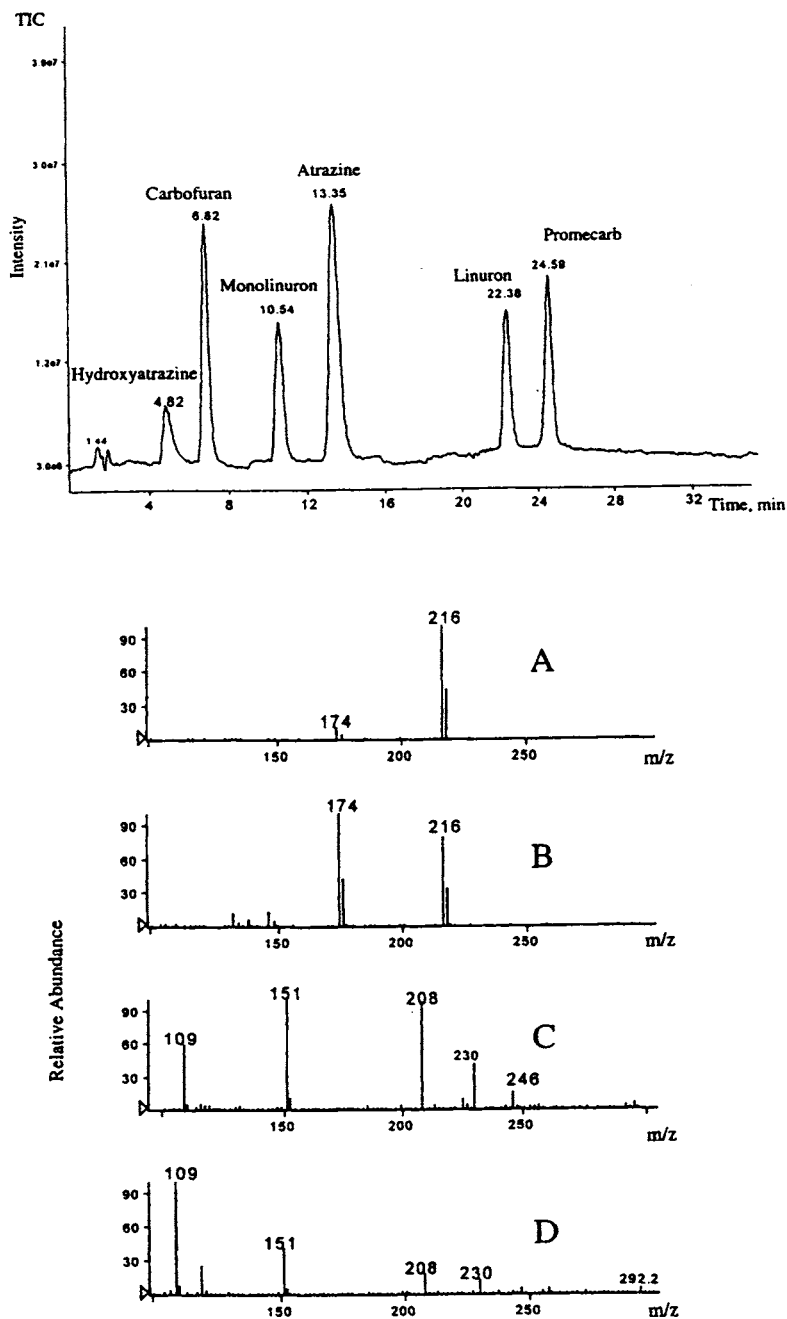
Fig. 20.21. LC/ISP–MS spectra of atrazine (panels A,B) and promecarb (panels C,D) recorded within the same run at orifice voltage of 20 V (panels A, C) and 60 V (panels B,D). Ion identity: atrazine, m/z 216 [M + H]$^+$, m/z 174 [M + H – C(CH$_3$)$_2$]$^+$; promecarb, m/z 208 [M + H]$^+$, m/z 230 [M + H]$^+$, m/z 151 [M + H–O=C=N–CH$_3$]$^+$, m/z 109 151$^+$–C$_3$H$_6$ [210].

MS system. The authors state that, when analysing 'clean' water samples, no benefit accrued from using the more specific detection of LC/ESP–MS/MS. The authors report stable performance of their system and no need for maintenance for over 6 months.

In a series of recently published papers Hogenboom et al. [97,174,216] reported a novel approach (cf. above) to multiresidue analysis of micropollutants in water samples. The analytes are enriched and then separated on a single-short-column (SSC), which is typically 10–40 × 2–4 mm i.d. The method is used especially in target screening where a significant gain in analysis time, i.e. 15 min (SSC) instead of 60 min (on-line SPE/LC [121]) per sample can be achieved by reducing the selectivity of the LC separation step while using (tandem) MS for sensitive detection. An extreme example of low resolution of ten phenylureas is shown in Fig. 20.22. In earlier work [174], the performance of SSC/APCI–MS/MS was tested with a mixture of 17 pesticides, which included organophosphates, carbamates, phenylureas and triazines. Using 2-cm long columns and 15 ml of surface or tap water, detection limits were 0.03–5 μg/l in the full-scan mode and 2–750 ng/l in SIM. The in-house compiled library of product-ion spectra from protonated molecules [121] allowed rapid confirmation of selected pesticides at a level of 0.1 μg/l in tap water and identification of real-world unknowns in surface water.

In another study [216], a SSC/APCI-MS/MS triple quadrupole system for the target analysis of six triazines and eight phenylurea herbicides was described. Commercially available 1 cm × 2.0 mm i.d. cartridges packed either with 8 μm C-18-bonded silica or 10-15 μm PLRP-S copolymer sorbent were used for SSC. Since they fit well into the PROS-PEKT (automated sample preparation unit), the exchange of used cartridges, i.e. SSC columns, is possible in a fully automated way. Using MS/MS in the multiple reaction monitoring mode (two product ions for each individual precursor ion selected), the detection limits of all compounds were 30–200 pg injected. Analysis of 4-ml river water samples allowed detection of all analytes at concentrations of 10–100 ng/l, which is 10–50 times below the threshold levels typically laid down by regulatory bodies. Analysis of river Rhine water showed the presence of simazine, atrazine, terbutylazine and diuron at concentrations of 20, 180, 30 and 80 ng/l, respectively. SSC cartridges showed good stability and could be re-used for at least 40 analyses. The ruggedness of the system was demonstrated by a series of overnight runs. Comparing the results with a similar TSP–MS/MS set-up from the same laboratory [172], the authors showed that the state-of-the-art APCI–MS/MS system provides ca. 10-fold improved detection limits, which allowed appropriate reduction of the sample volumes, i.e. a significant reduction of the analysis time.

In a follow-up study, the SSC set-up was coupled to APCI/ion-trap tandem MS instrument [97]. This allowed the detection of all analytes at 0.1–1 μg/l levels, and identification of 80–100 ng/l atrazine in several surface water samples was possible. An interesting comparison of quantitative data with those obtained using the same detection modes (scanning product ion, SRM) on a triple-quadrupole MS/MS instrument [216], showed ca. 10–30-fold lower sensitivity of the LCQ ion-trap (Table 20.9). Calibration curves obtained with the ion trap were linear in the range of 0.5–10 μg/l; however, repeatability of phenylurea analyses at the 1 μg/l level was somewhat poor (32–85%; $n = 15$). These results are less good than those obtained with the triple-quadrupole system. The authors try to explain this difference on the basis of various processes which occur within the ion trap, and the measurement procedure itself. The deliberately poor chromatographic resolution

Fig. 20.22. Schematic representation of single-short-column set-up (top) and single-short-column LC/APCI–MS/MS of loop injection of a mixture of ten phenylurea herbicides (125 ng of each; bottom). (A) full-scan TIC chromatogram and (B) the product-ion monitoring of [M + H]⁺ ions [174].

TABLE 20.9

COMPARISON OF ANALYTICAL DATA OF SHORT-COLUMN-QqQ (TRIPLE-QUADRUPOLE MS/MS) AND SHORT-COLUMN-ION TRAP MS/MS [216]

	Triazines		Phenylureas	
	QqQ	Ion trap	QqQ	Ion trap
Detection limits (pg; loop injections)	30–100	100–200	30–200	1000–2000
Detection limits (ng/l, SSC); 4 ml sample)	10–30	100–200	10–100	500–1000
Repeatability (4 ml sample)	At 0.5 μg/l: RSDs < 5% (n = 10)	Not tested	At 0.5 μg/l: RSDs < 4% (n = 10)	At 1 μg/l: RSDs 32–85% (n = 15)

on the SSC prohibits the use of time-scheduled product-ion or SRM procedures, which in its turn, leads to somewhat poorer detection limits. From among the MS/MS operational parameters it was found that the long interscan times, required between monitoring various precursor-product ion transitions, play a significant role in the procedure with the LCQ. In addition, when there are large differences in m/z values between precursor ion and product ion, this results in less efficient storage of product ions. An additional feature, typical of the LCQ ion-trap instrument, multiple-stage MS/MS (MS^n), was shown for propazine breakdown (Fig. 20.23). The product-ion mass spectra from the ion trap (only one major product ion in the first stage for triazines) differ from those obtained with the triple-quadrupole instrument, which may be a disadvantage when more general screening is pursued. The possibility of SRM over a number MS/MS stages has not yet been studied.

The analysis of pesticides with ESP-ITMS was reported by Lin et al. [96]. When using in-source CID or MS/MS CID, full-scan ESP spectra could be obtained for 10–30 pg of compound, which corresponds with low-μg/l detection limits in spiked water samples. The product ions from in-source CID and MS/MS CID of aldicarb sulfone were shown to be identical, although the signal-to-noise ratio of the MS/MS spectra was much better (Fig. 20.24). This stresses the point that the LC separation should be such that only single compounds are introduced into the ESP, in order to obtain useful in-source CID spectra.

A general approach for the characterisation of polar organic pollutants in surface water was reported by Schröder [217]. The author used both ESP and APCI with MS or MS/MS detection for analysis of Elbe and Saale river extracts. FIA/MS was used to obtain an 'overview spectrum' while FIA/MS/MS was used to characterise classes of compounds by monitoring parent-ion and neutral loss scans. In the next step, individual compounds were confirmed/identified from their product–ion spectra. Next to aromatic sulfonic acids, numerous industrial pollutants, e.g. anionic and non-ionic surfactants, nonylphenolethoxylates, polyethyleneglycols, alkylphosphates, alkylbenzenesulfonates and phthalates were detected.

Kawasaki et al. used LC/APCI–MS with a magnetic sector instrument for the analysis of 21 organophosphorus [98] and eight carbamate [218] pesticides in blood from
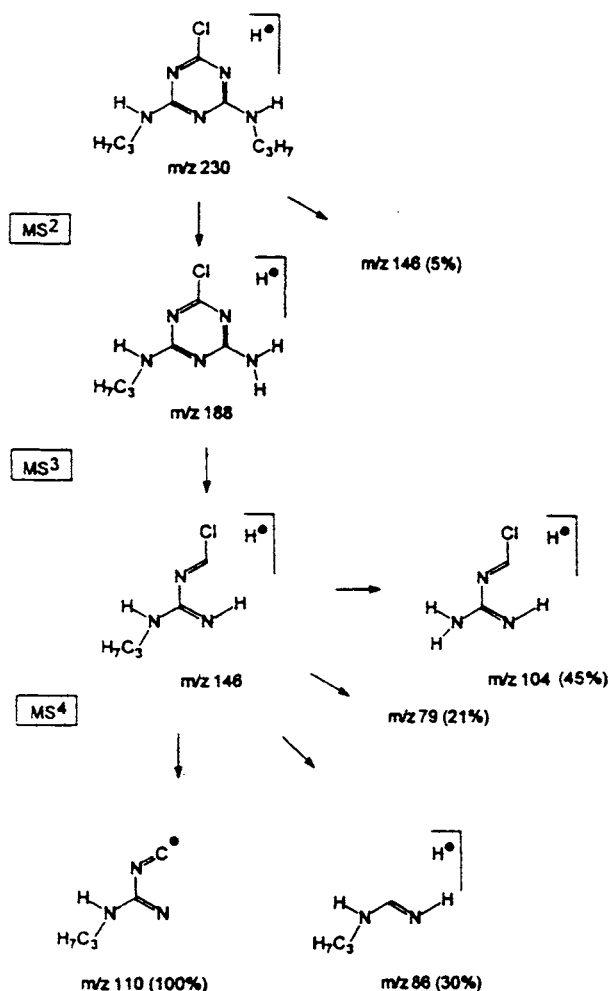
Fig. 20.23. Breakdown of propazine in multistage MS/MS in an ion-trap mass spectrometer [97].

patients suffering acute poisoning. LC separation was obtained on a C-18 column, with a methanol/water gradient and ammonium acetate as additive. The addition of higher levels (>10 mM) of ammonium acetate was shown to cause a 10-fold reduction of the sensitivity of detection for most compounds. Unequivocal identification of the pesticides could be achieved with both PI and NI detection. The instrument limits of detection, as determined from SIM experiments, were 2–20 ng (100 μl injections) for organophosphorus compounds best amenable to PI detection, and 2–1000 ng for organophosphorus compounds best amenable to NI detection. The use of the PI mode, with SIM detection of the $[M + H]^+$ ions, led to instrument detection limits of 12–60 ng for the carbamates. Compounds with a molecular weight below 200 amu were found to suffer from baseline instability, which caused less sensitive detection. Preventing cluster ion formation, or cluster ion breaking, may lead to improved detection perfor-
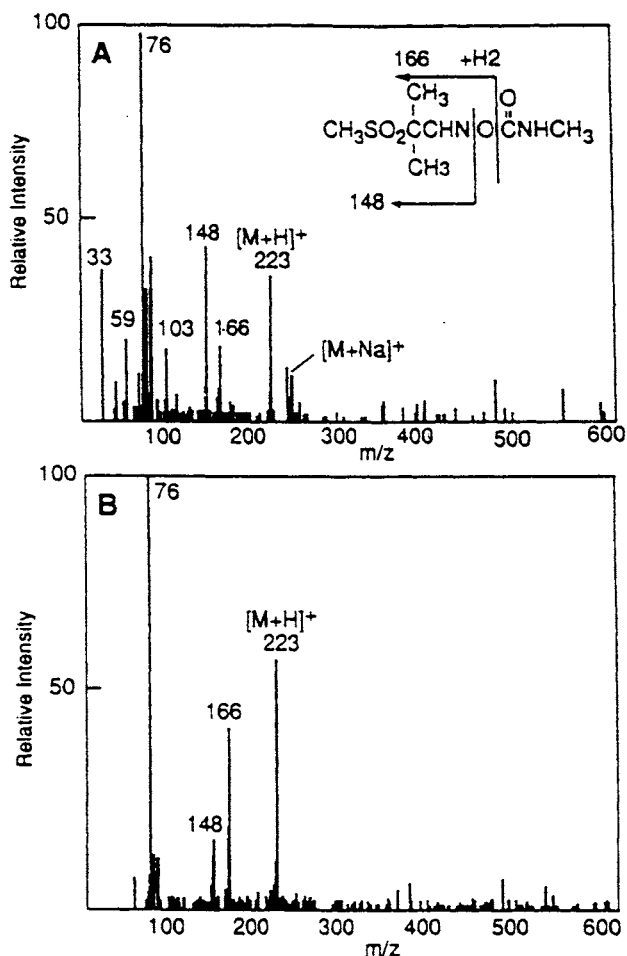
Fig. 20.24. ESP CID spectra of 1 ng of aldicarb sulfone (mol. wt. 222): (A) CID spectrum obtained in the ESP in-source region at a repeller setting of 15 V; (B) CID spectrum of the [M + H]$^+$ ion (*m/z* 223) obtained in the ion trap [96].

mance. The authors noted that quantification by means of LC/APCI–SIM–MS is as good as that of GC/MS, but that the sensitivity of full-scan qualitative analysis is inferior to that of GC/MS.

ISP–MS in conjunction with immunoaffinity trapping was used by Rule et al. [219] for the highly selective determination of carbofuran. An immunoaffinity precolumn was employed for selective enrichment of the analyte; trapping was followed by desorption and on-line LC separation and MS detection. The whole procedure was automated by means of a gradient controller. Full-scan detection limits were 40 ng/l for spiked surface water and 2.5 ng/g for a crude potato extract. The selectivity towards carbofuran was demonstrated in experiments dealing with the coelution of fluometuron: this compound could not be observed when using the immunoaffinity precolumn. A similar methodology

was used for carbendazim in soil extracts and lake water [220]. The 33×2.1 mm i.d. immunoaffinity precolumn packed with Hi Pack Protein G sorbent was loaded first with 20 μg of antibody followed by a sample. After elution, carbendazim was retained selectively on a trapping precolumn packed with semipermeable C-18-bonded silica and then eluted on-line onto the analytical column. Detection was accomplished by ESP–MS/MS, the analyte was monitored by selected reaction monitoring (m/z 192 ion of protonated molecule to product ion m/z 160). The complete set-up was automated by means of an INTEGRAL Micro-Analytical Workstation. Detection limits were 25 ng/l and 100 μg/kg of carbendazim in lake water and soil samples, respectively, which is 100-fold better than can be obtained with UV detection.

Automated off-line SPE on cartridges packed with an anti-isoproturon immunosorbent followed by LC/DAD UV/APCI–MS was used by Ferrer et al. [221] for the selective determination of chlorotoluron, isoproturon, diuron, linuron and diflubenzuron in LC-grade water and groundwater. Limits of detection obtained by APCI–MS under SIM conditions (except for diflubenzurone, by monitoring the ions of the protonated molecules) were 0.6–1 μg/l, which is similar to those obtained by LC/DAD UV. APCI–MS, however, allowed resolving of the coeluting isoproturon and diuron. Recoveries ranged from 16% to 97%, when using 50-ml samples spiked with 3 μg/l of each analyte. The method was satisfactorily validated in an inter-laboratory study using Aquacheck certified samples [205].

In a follow-up study [222] the authors combined immunosorbent SPE and LC/MS on-line (OSP-2A) to determine seven triazines and their degradation products and seven phenylureas. Enrichment of 20-ml natural water samples on a cartridge packed with anti-atrazine or anti-chlorotoluron antibodies permitted to reach detection limits of 1–5 ng/l. The method was applied also for the analysis of sediment samples, which were first Soxhlet extracted with methanol and then brought to volume and enriched as above. APCI–MS was operated in the PI mode, using TS-SIM and deuterated atrazine as an internal standard for quantification. All compounds were measured at a CID voltage of 20 V, since the increase to 40 V did not result in enhanced fragmentation but caused a decrease in sensitivity. The immunoaffinity column life-time was from 3 to 6 months up to 2 years.

The automated on-line SPE (OSP-2A) and LC/ESP–MS in NI mode was used also for analysis of eight acidic herbicides in estuarine water [223]; detection limits in SIM mode were below 30 ng/l.

An increasing number of papers deals with applications of LC/API–MS for the identification of degradation/transformation products of pesticides in environmental water samples. Lacorte et al. [224] studied two organophosphorus pesticides, fenthion and temephos, which were oxidised with N-bromosuccinimide. Next to LC/APCI–MS also NMR and LC/DAD UV were applied for identification of the oxidation products. When using an APCI extraction voltage of 40 V, more than three different fragment ions were generated for each parent compound and transformation product, which permitted their unequivocal identification and/or confirmation. Four transformation products were detected and identified for fenthion, and six for temephos. The analysis of a real-world estuarine water sample contaminated with temephos showed the presence of four transformation products. Compared to their previous study using TSP–MS and the same compounds [225], it is obvious that the APCI–MS spectra provide more structural infor-

mation. LC/ESP–MS/MS was used for the identification of atrazine derivatives produced by Fenton's reagent [226] and for the confirmation of the presence of hydroxylated degradation products of atrazine in river water [227].

Seven carbamates and three of their degradation products were analysed in extracts of strawberries and plums by LC/APCI–MS [228]. The authors used a PI/NI switch during data acquisition. This enabled simultaneous determination of eight analytes in the PI and two analytes in the NI mode. The base peak for most compounds was the $[M + H]^+$ ion, except for aldicarb, which showed the highest signal at m/z 116 ($[MH-75]^+$) corresponding to the loss of carbamic acid. Under NI conditions the base peak of diflubenzuron was $[M - H]^-$ and that of clofentezine, was $[M]^{-\circ}$. When processing 30 g of sample with ethyl acetate, SIM detection limits were 0.002–0.025 ng/μl in solvent-based standards (equivalent to 0.002–0.033 mg/kg in the crop). A reporting limit of 0.02 mg/kg was set, which is ca. 5–150-fold lower than current Codex Alimentarius Commisssion maximum residue levels [229]. The responses of the analytes were linear within the working range of 0.025–0.5 ng/μl, except for carbofuran and 3-hydroxycarbofuran; this was due to their ca. 3-fold lower sensitivity. Suppression or enhancement effects were observed for most compounds. They were both matrix- and compound-dependent. The LC/APCI–MS was used also for analysis of diflubenzurone in mushrooms [230].

Off-line SPE using the increasingly popular GCB sorbent followed by pneumatically-assisted ESP–MS was used for the analysis of 45 wide-polarity range pesticides and their degradation products [231]. In the method, drinking water (4 l), groundwater (2 l) and river water (1 l), was passed through a 0.5-g GCB cartridge. Recoveries of all analytes were above 75%. The method provided an excellent detection limits, e.g. in drinking water 0.06–1.5 ng/l in the SIM mode and 1–9 ng/l in the full-scan mode. For groundwater and river water the limits were about 2-fold and 4-fold higher, respectively. To eliminate traces of Na^+, the authors used in-glass distilled methanol and acetonitrile. In a continuing discussion on the effects of additives to the eluent, the authors optimised the addition of trifluoroacetic acid to 10 μmol/l in both eluent constituents. Further increase caused a reduction of the $[M + H]^+$ signal, which is in contrast with results of other authors, who observed a steady increase of the $[M + H]^+$ signal for increasing formic acid concentration up to 25 mmol/l, i.e., ca. 2.5 mmol/l proton concentration [208]. This work seems to be the only recent systematic study which deals specifically with the effect of the concentrations of various additives in the eluent on the ESP–MS detector response for non-ionophore analytes. Major ions obtained for three different in-source CID voltages were reported. It was possible to obtain structure-specific information for all analytes without relevant loss in sensitivity. The authors suggest to perform two analyses of the same extract at two different CID voltages to obtain as specific information as possible. The intra- and inter-day variations of the signal intensities were 1.5–4.1% and 7.8–10.2%, respectively, and the method was found to be robust. The method was successfully applied to the analysis of atrazine and its six major degradation products [211]. In the full-scan mode, detection limits varied from 40 to 300 ng/l in river water samples. Inter-day reproducibility was less than 10% in the full-scan (25 ng/l level) and the SIM (3 ng/l level) mode.

A GCB extraction of water samples was also used prior to another LC/ESP–MS method for the analysis of atrazine and hydroxyatrazine using a microbore (50 × 0.1 mm i.d.)

analytical column [232]. Quantification was based on the addition of isotopically labelled internal standards, providing RSDs of peak areas at 100 ng/l (atrazine) and 50 ng/l (hydroxyatrazine) of less than 15% ($n = 6$). The instrument SIM detection limits were 10 pg and 30 pg for atrazine and hydroxyatrazine, respectively. The appearance of sodiated molecules and dimer ions in mass spectra was considered to be an advantage because of the additional molecular mass information. The method was applied to the analysis of agricultural run-off water samples; the target compounds were found at levels of 1.9–13.2 µg/l. Compared to previous work with high-resolution (HR) GC-MS and HR–FAB–MS, LC/ESP–MS provided similar results. However, the instrumentation was much less complicated and expensive.

Off-line SPE using an ASPEC XL system prior to LC/ESP–MS was applied for automated analysis of ten organophosphorus pesticides [233]. A variety of SPE materials (Amberchrom, LiChrolut EN, cyclohexyl, styrene-divinylbenzene copolymer, C-18-bonded silica, ISOLUTE ENV) was used for the isolation of the analytes from 200-ml ground water samples. The LC separation was accomplished on a cyanopropyl-modified column. The SIM detection limits obtained by monitoring $[M + Na]^+$ ions were 0.01–0.2 µg/l. The method showed the presence of two phthalates originating from the SPE cartridge material and fenthion sulfoxide, a transformation product of fenthion. It was shown that cis- and trans-mevinphos can be distinguished on the basis of a specific ion at m/z 99 for the trans isomer.

The presence of sodium adducts is a serious issue in ESP–MS analysis. Sodium ion addition is, apparently, thermodynamically favoured and occurs preferentially in dilute solutions, as shown for, e.g. sulfonylureas [90]. One should be also aware that in ESP–MS several groups of compounds (e.g. phenylureas [211] or organophosphorus pesticides [233]) exhibit very stable $[M + Na]^+$ ions and do not fragment even at high in-source CID voltages, which is in contrast with the behaviour of, e.g., triazines [211]. As regards the presence of sodiated molecules (also see refs. [90,163,211]), it is concluded in ref. [233] that their formation (i) is related to the use of methanol in the eluent, (ii) is compound dependent and (iii) is dependent on the concentration of the analyte and on the cone voltage used. Indeed, the use of acetonitrile in the eluent decreases the abundance of sodiated ions, as reported for organophosphorus pesticides [234], and monuron and carbofuran [86]. Also, acidification [213,214], addition of NH_4^+ into the eluent or in-glass methanol distillation [235] cause a significant decrease of signal intensity of the sodium adducts. As regards the widespread use of trifluoroacetic acid or other volatile strong acids in the LC/ESP–MS eluent, one should be aware that they cause suppression for basic compounds. This is due to strong ion pairing between the TFA anion and the protonated sample cation of the basic analytes. After having been rendered 'neutral', the molecules are then 'masked' from the ESP–MS electric fields. A practical approach to enhance the signal intensity for most basic analytes ca. 10–50-fold is the post-column addition of a solution of 75% propionic acid and 25% isopropanol in a ratio 1:2 to the column flow [236].

cf-FAB and ESP were combined with a magnetic sector instrument for the quantitative analysis of four sulfonylurea herbicides [90]. NI–ESP led to the successful identification of a sulfate conjugate of hydroxylated bromacil in goat urine. In all analyses, LC band broadening was observed for cf-FAB, but not for ESP. cf-FAB and ESP yielded similar spectra for the compounds studied: both ionisation methods generate prominent quasi-

molecular anions or cations. However, ESP spectra were more sensitive to changes in the LC eluent composition and analyte concentration than FAB spectra. Especially the metal adduct ions, e.g. $[M + Na]^+$ and $[M + K]^+$, and cluster ions, e.g. $[2M + Na]^+$, which are observed with both methods, adversely affect quantification and the interpretation of spectra of unknowns. Amounts of 1–10 ng (1–100 mg/l with the 0.1–1 μl injection volumes used) of the sulfonylureas could easily be detected with both ionisation modes, provided that an MS-array detector was used instead of a common electron multiplier. Both techniques allowed accurate HRMS measurements but with cf-FAB structure elucidation was more straightforward.

The identification of organotin pesticides has been studied by ESP–MS and ISP–MS. These pesticides have not been analysed by any of the other LC/MS methods, because they are often amenable to GC/MS (or GC/AED). Tributyltin compounds were extracted from sediment and the extracts were directly subjected to FIA/ISP–MS/MS. Selected reaction monitoring (SRM), by scanning the loss of two butene molecules from the tributyl-^{122}Sn$^+$ isotopomer ion, was found to provide sufficient selectivity for reliable quantification [237]. The detection limit for tributyltin compounds was established at 5 pg (0.2 mg Sn equivalent per kg of sediment). A wide variety of alkyl- and aryltin compounds was studied by Jones and Betowski, who used LC/ESP–MS [91]. Specific fragmentation and acetate adduct ion formation (acetate coming from the acetic acid eluent additive) generally allowed unequivocal identification of the analytes. The published data show that LC/ESP–MS has a good potential for the detection of organotin compounds; however, no detection limits were reported.

A more fundamental study on the determination of CID pathways and cross-sections of 19 organophosphorus compounds by APCI–MS/MS was presented in ref. [238]. A generalised scheme for dissociation of the studied classes of analytes was derived from CID experiments and a simple model for the determination of CID cross-sections was developed. APCI–MS/MS was thus shown to be a useful diagnostic tool in the analysis of organophosphorus compounds.

Ten carbamate pesticides were analysed by LC/TSP–MS and LC/ISP–MS in water and sediment samples [239]. It was shown that absolute detection limits obtained with LC/ISP–MS were in the 10–60 pg range, which is 10–150-fold better than those obtained with LC/TSP–MS.

In a review, Voyksner summarised the potential of LC/API–MS for environmental analysis [15], using the determination of carbamates and aromatic amines by means of LC/APCI–MS and LC/ESP–MS as examples. The possibility of efficient ionisation and gaining structural information by in-source CID is a main advantage of the API-based techniques. The author concludes that acidic and basic compounds are most effectively analysed by LC/ESP–MS, while less polar compounds are better amenable to LC/APCI–MS. A general overview of API–MS instrumentation, techniques and selected applications is given in [11,12,20,21], and a review discussing applications of on-line SPE/LC/MS is given in [240]. A discussion on LC/MS analysis of carbamates, especially carbofuran, is presented in [241].

The performance of pneumatically assisted (PA)-ESP and APCI interfaces for the LC/MS analysis of 73 wide-polarity-range pesticides was studied by Slobodník et al. [121]. The authors used various modes of operation including PI, NI, PI/NI switching during the run, MS and MS/MS. The whole set-up was automated by means of on-line coupling of
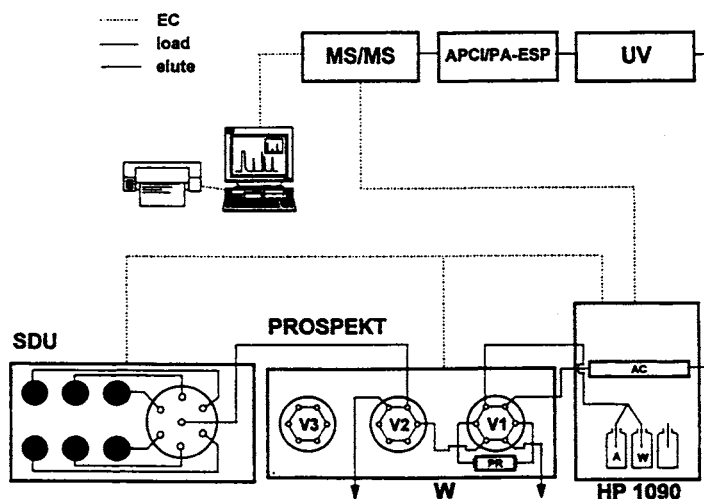
Fig. 20.25. Schematic representation of the automated on-line SPE/LC/API–MS/MS [121].

SPE/LC and API–(MS)MS using a PROSPEKT-sample handling device (Fig. 20.25). For a selected group of 17 pesticides detection limits obtained with 100-ml spiked tap water samples by either technique were 0.007–3 μg/l in the full-scan mode and 0.1–200 ng/l under SIM conditions (Table 20.10). Two pesticides, fenchlorphos and bromophos-ethyl, could not be detected at all. In general, APCI mass spectra showed less sodium adduct ions, and allowed detection of a wider range of compounds, the less polar ones being included. In addition, operation at flow rates up to 1.5 ml/min was permitted (optimum for PA–ESP, ca. 100–300 μl/min). NI operation was recommended for the target analysis of, e.g., chlorophenols, nitrophenols and several organophosphorus pesticides and phenylureas. PA–ESP–MS/MS and APCI–MS/MS gave similar product-ion spectra from protonated molecules (Fig. 20.26) and an MS/MS library was built for more than 60 analytes. It has been successfully used for searching product-ion spectra at low concentration levels (10 ng/l; Fig. 20.27) in tap water and the identification of atrazine in surface water (250 ng/l). With dimethoate as example, it was found that APCI–MS/MS, PA–ESP–MS/MS, TSP–MS/MS and GC/PCI–MS/MS spectra of protonated molecules obtained in different laboratories are rather similar.

As regards the serious difficulties often encountered when trying to interpret MS/MS spectra for the identification of unknowns, the idea of building MS/MS libraries will certainly be an issue in further API–MS/MS developments. Recently, a standardised procedure for the extraction of relevant product-ion spectra (from protonated molecules) and their systematic storage in libraries was proposed by Kienhuis et al. [122].

There is obviously also a need for establishment of API–CID–MS spectra libraries, which has been recognised by manufacturers of analytical instrumentation. Since different molecular ions have different collision stabilities, it was found that rotating the energy of the API–CID on alternate scans and averaging the spectra across the chromatographic peak produced spectra most suitable for library building [123].

TABLE 20.10

ESTIMATED DETECTION LIMITS (SIGNAL-TO-NOISE 3) OF 17 PESTICIDES OBTAINED FROM SPE/LC/PA-ESP–MS AND SPE/LC/APCI–MS IN FULL-SCAN, SIM AND MS/MS MODES [121]

No.	Compound	SPE/LC/PA–ESP–MS		SPE/LC/APCI–MS			Class
		100 ml					
		Full-scan (ng/l)	SIM (ng/l)	Full-scan (ng/l)	SIM (ng/l)	MS/MS (ng/l)	
1	Oxamyl	500	3	500	3	n.m.	Carbamates
2	Dimethoate	30	1	20	1	7	Organophosphates
3	Aldicarb	100	2	25	2	n.m.	Carbamates
4	Monuron	40	2[b]	10	0.6	3	Phenylureas
5	Propoxur	30	0.5	30	1.5	n.m.	Carbamates
6	Diuron	300	2[b]	20	0.6	8	Phenylureas
7	Propazine	8	0.1	7	0.4	1	Triazines
8	Terbutylazine	10	0.1	8	0.4	1	Triazines
9	Fenamiphos	10	0.2	10	0.8	1	Organophosphates
10	Alachlor	50	3	60	6	30	Anilides
11	Neburon	40	2	20	1.5	4	Phenylureas
12	Fenthion	n.d.[a]	n.m.[a]	3000	n.m.	n.m.	Organophosphates
13	Coumaphos	500	20	350	15	75	Organophosphates
14	Fenchlorphos	n.d.	n.m.	n.d.	n.m.	n.m.	Organophosphates
15	Chlorpyriphos	n.d.	n.d.	2500	150	n.m.	Organophosphates
16	Trifluralin	n.d.	n.d.	3000	200	n.m.	Nitrophenols
17	Bromophos-ethyl	n.d.	n.m.	n.d.	n.m.	n.m.	Organophosphates

[a] n.m., not monitored; n.d., not detected at highest concentration analysed.
[b] SIM detection limits obtained in the NI mode.
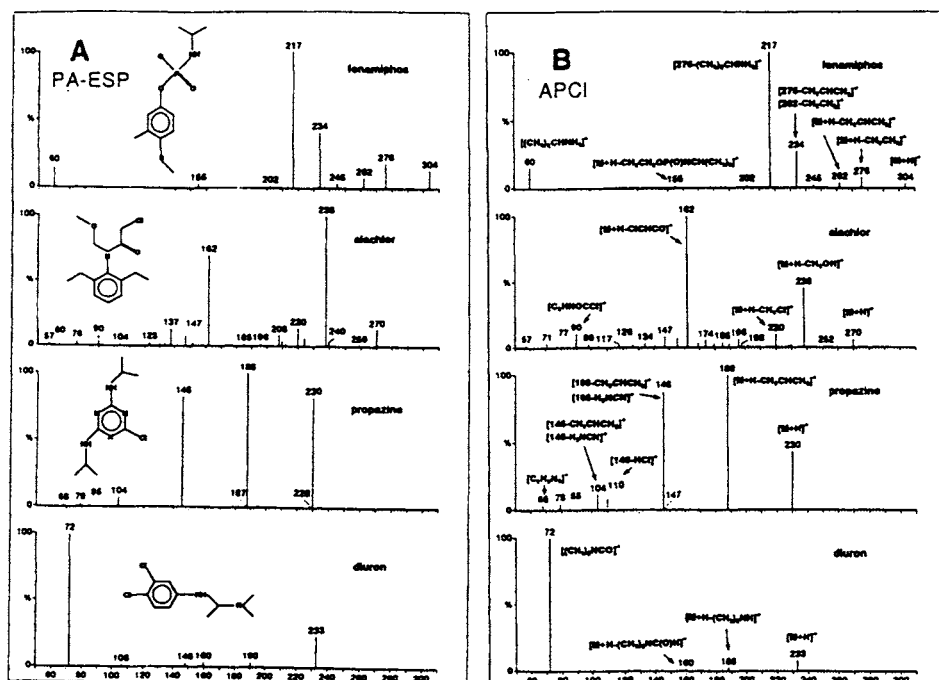
Fig. 20.26. Product ion spectra obtained from protonated molecules of fenamiphos, alachlor, propazine and diuron in (A) PA–ESP–MS/MS and (B) APCI–MS/MS. The APCI experiment was performed with injections of 125 ng of each compounds into the SSC, temperature of the ion source was 150°C, flow rate of mobile phase 1 ml/min; the PA–ESP–MS/MS spectra were obtained without chromatographic separation within continuous infusion of 5 mg/l solution of the analytes at flow rate of 25 μl/min, temperature of the ion source was 70°C [121].

20.4 CONCLUSIONS

In the past few years, LC/MS has became a generally accepted and widely applied technique. Admittedly, in the field of environmental analysis, its use in routine laboratories still is somewhat limited, but the increasing interest for polar pesticides and industrial chemicals, and their more polar (bio)degradation products indicate that more widespread use for routine purposes can be expected in the near future. The present review shows the potential of a variety of LC/MS techniques for the trace-level identification and/or quantification of analytes covering a wide polarity and volatility range. It also shows that analytical chemists are increasingly aware of the fact that the proper choice of the LC/MS technique to be used in a specific case depends on the analyte class that has to be studied, the sample type offered for analysis and, last but not least, the goal one has in mind: the detection of target or, also, of unknowns, the identification and/or confirmation, and/or quantification. Currently, instruments typically vary from relatively inexpensive benchtop LC/MS machines, which are built primarily as a selective detector for LC, to sophisticated LC/tandem-MS machines with a variety of options and modes of operation.
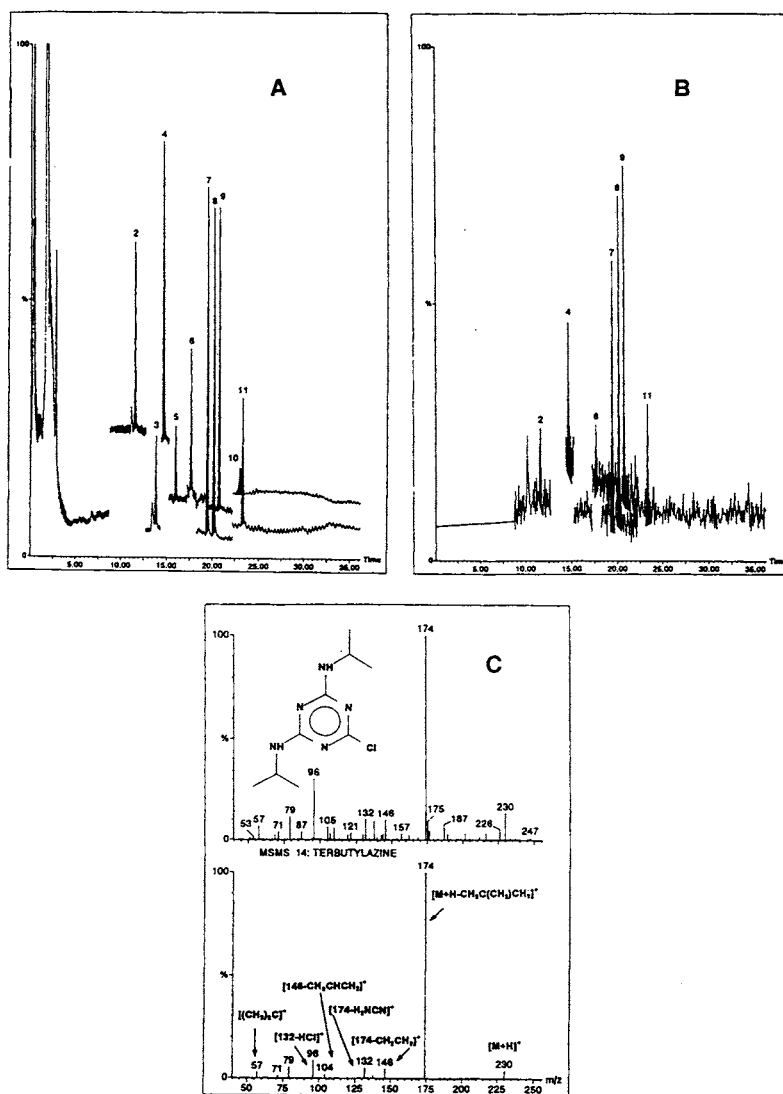
Fig. 20.27. Chromatograms of 100 ml tap water spiked with the mixture of 17 pesticides, obtained by (A) SPE/LC/APCI–MS in TS-SIM (monuron and diuron monitored in NI mode) and (B) SPE/LC/APCI–MS/MS. Concentration of each analyte is 10 ng/l (for numbers, see Table 20.10). Product ion (MS/MS) spectrum of terbutylazine (8) at 10 ng/l level is compared to MS/MS spectrum of the standard compound obtained from 125 ng injection (C) [121].

All of these are easy to control by integrated software and can be used for fully automated, unattended operation.

Despite satisfactory analyte detectability and confirmatory power, LC/TSP–MS is gradually becoming an obsolete technique from the scientific point of view, and is rapidly replaced by LC/APCI–MS (Fig. 20.28). A major drawback of LC/TSP–MS is the limited
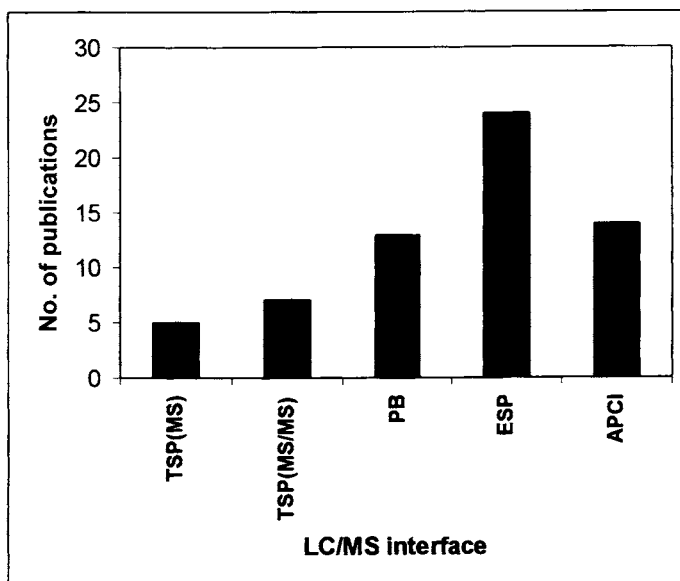
Fig. 20.28. Number of scientific papers published since 1995, related to the analysis of polar pesticides by LC/MS with TSP, PB, ESP and APCI interfaces.

structural information provided by the mass spectra (although one should add that the information by API techniques often also leaves much to be desired). Several studies have been published which show that coupling of LC and TSP–MS/MS is a good means to obtain relevant structural information. Optimisation of such a system is, however, rather complicated–which makes the set-up less robust.

It is most certainly true that essentially all of the eagerly sought GC/EI–MS-type structural information can be obtained in LC if a PB interface is used. However, even though its performance, in terms of sensitivity and linearity, has been improved over the years and quite a number of interesting applications have been reported, the PB interface has not really became popular because of the often poor sensitivity (which can only partly be compensated by efficient on-line analyte enrichment) and a rather capricious lack of response shown by many compounds of interest. Still, the general attractiveness of a PB-type interface should be kept in mind, with the simultaneous operation of LC/PB–MS and GC/MS sharing the same mass spectrometer, as an interesting example.

Without doubt, the most dramatic developments are today occurring in the field of API interfacing. Valued features are an excellent sensitivity (with instrument detection limits in the SIM mode at the low-pg level) and the possibility to obtain additional structural information by in-source CID. Several API–MS instruments are now commercially available. LC/ESP–MS is well suited for the study of polar analytes containing an acidic or basic group, while less polar compounds can best be addressed by means of LC/APCI–MS. Because of a different ionisation mechanism APCI–MS is less dependent on the solution chemistry (sample and eluent pH adjustment prior to analysis is often required in ESP–MS) and seems to be more suited for multiresidue pesticide analysis. Furthermore, there are less 'unwanted' sodium/potassium adduct ions formed in APCI–MS, i.e., the

quantification and CID fragmentation are more straightforward than with ESP–MS. So far almost all API–MS applications for 'small' environmental molecules are target-analysis oriented. Therefore, in the near future one may well find that more effort will be put into exact mass measurements to facilitate the identification of unknowns, e.g. by the coupling of API interfaces to more expensive instruments such as sector MS, reflectron TOF-MS or FT-ICR-MS.

In most instances, the sensitivity of the current LC/MS techniques does not allow the direct analysis of samples without any pretreatment. Fortunately, it has by now been convincingly demonstrated that combining off-line or, preferably, on-line SPE and LC/MS allows low-ng/l detection limits to be achieved for aqueous samples, for all the interfacing techniques reviewed. On-line SPE/LC/MS is being used increasingly because of the possibility to automate the total analytical set-up, and to significantly reduce the time of analysis. The use of this approach for pesticide degradation studies merit special attention. A most interesting recent development is the use of the 'single-short-column' technique which allows analyte enrichment and LC analysis on a single 1–2-cm long column, with a total time of analysis of about 10 min. The more efficient procedure enables a significant reductions of sample volumes without noticeable loss of sensitivity.

Finally, it is clear by now that MS/MS methods, coupled to any of the reviewed interfaces, offer distinct advantages in the field of structural studies, the characterisation of mixture components and trace analysis. Because of the difficulties often encountered when interpreting complex MS/MS spectra there have been several attempts to build libraries of MS/MS product-ion spectra from protonated molecules. A successful outcome will, no doubt, contribute to a rapidly increasing popularity of LC/MS/MS techniques. For the rest, although triple-quadrupole instruments are used in many published papers, it is clear that ion-traps, especially those coupled to API interfaces, are of much interest to LC/MS users. Major advantages are the lower price and the possibility to obtain MS^n spectra for structure elucidation.

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Chapter 21

Organometallic compound determination in the environment by hyphenated techniques

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CONTENTS

ABBREVIATIONS

AAS	atomic absorption spectrometry
AB	arsenobetaine
AC	arsenocholine
AED	atomic emission detection
AES	atomic emission spectrometry
AFS	atomic fluorescence spectrometry
CT	cryogenic trapping
D	derivatization
DCP	direct current plasma
DIN	direct injection nebulizer
DMA	dimethylic arsenic acid
ECD	electron capture detection
ES	electrospray
F	flame
FIA	flow injection analysis
FID	flame ionization detector
FPD	flame photometric detection

GC   gas chromatography
GF   graphite furnace
HG   hydride generation
HPLC   high-performance liquid chromatography
ICP   inductively coupled plasma
IE   ion exchange
IP   ion pairing
LC   liquid chromatography
LEI   laser-enhanced ionization
LEAFS   laser-excited atomic fluorescence spectrometry
MGLS   membrane gas–liquid separator
MLC   micellar liquid chromatography
MMA   monomethyarsonic acid
MS   mass spectrometry
QF   quartz furnace
PDC   pyrrolidinedithiocarbamate
PED   plasma emission detection
PTFE   polytetrafluoroethylene
RP   reversed phase
SeCys   selenocysteine
SeMET   selenomethionine
SFC   super critical fluid chromatography
TBT   tributyltin
TEL   tetraethyllead
TETRA   tetramethylarsonium ion
TMAO   trimethylarsine oxide
TML   tetramethyllead
TMSe   trimethylselenonium
USN   ultrasonic nebulizer
UV   ultraviolet.

21.1 INTRODUCTION

21.1.1 Organometallic compounds in the environment

The fate of trace metals and their impact on the environment have been a major concern for more than 25 years. Most studies have been limited to total metal determination. In general, organometallic compounds represent a small fraction of the total metal burden in the sample. However, organometallic forms of metals are usually much more toxic than their inorganic counterparts.

Organometallic compounds occur in the environment as a result of direct anthropogenic inputs or because they are naturally formed there. They are used in a wide variety of industrial processes, sometimes at the percent level. Some organometallic forms of mercury, arsenic and now tin have been observed to have efficient biocidal properties. This fact has largely been applied to the synthesis of a large variety of pesticides, leading then to direct introduction in the environment. Another well-known source is directly related to the widespread use of methyllead ions as antiknocking additives in gasoline, resulting in an important global dispersion of lead species.

Natural methylation in the environment is now well established for a number of elements. Mercury was one of the first cases studied owing to its methylation potential and the high toxicity associated with the final product. Natural alkylation of arsenic and tin

are now well-established facts. Analytical or chemical evidence of environment methyl-metal formation also exists for selenium, tellurium, and germanium. Antimony methylation is now questioned due to possible analytical errors. Lead methylation is still controversial.

Except when under the influence of direct anthropogenic inputs, organometallic compounds occur in the environment at very low, ultra-trace levels. The yield of natural methylation mechanisms is always small. As a result, methylated species represent only a few percent of the total metal concentration in water and sediments. However, through bioaccumulation processes in the food chain, methylated forms of metal (e.g. Hg) can represent up to 90% of the total metal concentration present in fish flesh. Their lipophilic properties allows them selectively cross biological barriers an hence lead to rapid accumulation in biological tissues.

21.1.2 Analytical solutions

Their behavior and impact on biological systems and fate in the environment is not generally different from that of organic contaminants such as pesticides or polycyclic aromatic hydrocarbon. Their partition coefficient are most of the time similar. However, the fact that they contain a center with a metal has lead in general the analytical community to consider them has inorganic moieties and applied analytical solutions used for inorganic analysis [1].

This approach has been under constant evolution during the last 25 years. Analytical solutions have most of the time relied on atomic detectors such as atomic absorption (flame and graphite furnace) after the use of selective organic extractions. The concurrent progresses of separation techniques such as gas or liquid chromatography has lead inorganic analyst to hyphenate these separation methods to atomic detectors. Both separative sciences and atomic detection methods have gained rapid evolution both in resolution capabilities and selectivity and sensitivity, respectively [2]. The rapid progress of inductively coupled plasma techniques (ICP) using either atomic emission spectrometry (AES) or mass spectrometry detection (MS) approaches has allowed better continuous up-take of the analytical eluent. The selectivity and sensitivity plasma sources and ICP/MS has resulted in the continuous development of liquid separation techniques compared to gaseous approaches. However, the hyphenation in both cases is still the limiting factor.

Hyphenated techniques allow none the less the possibilities to perform organometallic determination. They have, however, also multiplied the analytical steps in the procedure. We can say that part of the success of speciation techniques lies in the quality and efficiency of the interface design between the different stages of the analytical procedure. In most cases, analytical speciation schemes rely on the combination of three basic steps and their interfacing design at the instrumental level:

- analyte preconcentration;
- separation (chromatographic or differential concentration mode);
- selective detection (single or multi-elemental).

All techniques first require a preconcentration step. This procedure can be performed off-line from the instrumentation, or on-line as a part of the instrumentation set-up.

The first range of techniques using off-line preconcentration most often use standard

chromatographic procedures (gas or liquid). The sample injection mode requires only a small volume of sample (a few microlitres). For both the gas or liquid separation approach, a very important preconcentration step is required prior to injection. Interfacing is in general straightforward and the gaseous nature of the effluent can be easily adapted to various atomic spectrometry techniques such as atomic absorption, atomic emission, atomic fluorescence or even mass spectrometry. Liquid chromatography separation techniques are very powerful as they generally allow the derivatization stage to be by-passed and provide a large panel of possible chromatographic procedures enabling a larger range of organometallic compounds to be determined. However, the small amount of eluent delivered leads to an overall low sensitivity. Interfacing is more complex in this case since the liquid eluent must be transformed into the gaseous state prior to detection. This stage is technically difficult and usually leads to an overall loss in sensitivity (Fig. 21.1).

An on-line technique using direct interfacing between hydride generation methods, and combining simultaneously the preconcentration step by cryofocusing and later chromatography by gentle warming of the trap can be very easily interfaced with atomic absorption spectrometry or atomic fluorescence. Sample volumes are larger in this case and range from 10 to 1000 ml. Techniques derived from these procedures achieve the highest sensitivity and ease of operation (Fig. 21.1) in comparison to other methods using off-line pre concentration methods with considerably fewer analytical steps. However, they are limited to low boiling point organometallic species (methyl, ethyl, butyl) and have a low resolution capacity. Nonetheless, the high sensitivity of these techniques has indicated the occurrence of unexpected species in the environment. In general, absolute detection limits of these hyphenated systems are always at the picogram level since they classically represent the detection limits achieved with the atomic spectrometry detectors. In all cases,
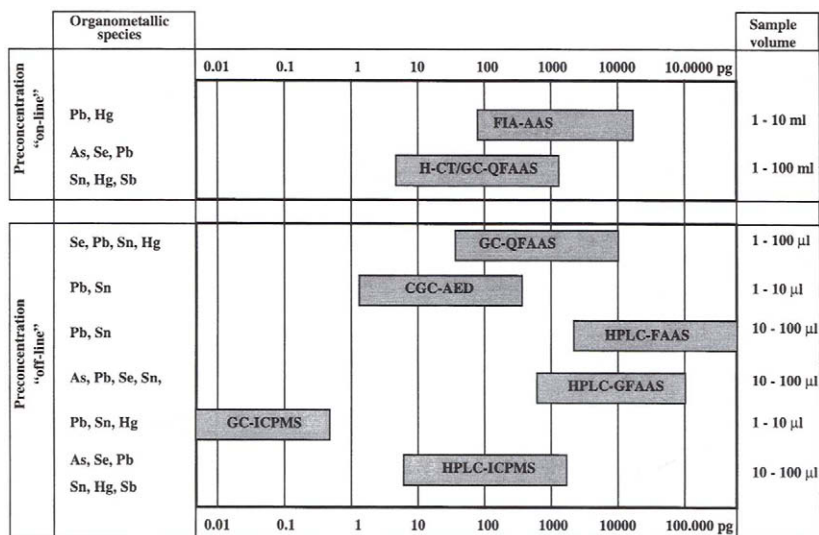
Fig. 21.1. Detection limit ranges of speciation techniques coupled to atomic spectroscopy detectors (in pg).

the quality of the preconcentration step is of vital importance since it then provides the suitable sensitivity for working conditions associated with environmental analysis.

21.1.3 The issue of speciation

Evolution towards metal speciation is not easy since the analytical market is well established and renewing itself with more sensitive equipment. Regulations follow these trends and are logically relying on the most common information available delivered by inorganic instrumentation. This feed back loop has slowly created the gap between rational decision based on metal species determination and a simplified approach using total metal determination. Speciation analysis does not only require good mastery of the spectroscopy and its fundamental physics, it also requires controlled, reliable and reproducible chemistry which is a fundamental prerequisite prior to spectroscopic detection. This part of the whole analytical system is more complex to control and has also prevented routine developments.

Indeed, organometallic species of Hg are far more toxic than their inorganic counterparts. Trialkyltin compounds can be considered to be some of the most toxic biocides synthesized and directly released in the environment.

Inorganic tin is nontoxic and is present in the tin can containing food. Legislative attitudes are ambiguous due the difficulty of obtaining reliable information by lack on routine instrumentation. This ambiguous position is well translated in a recent EEC regulation. EEC regulation 76/464 recommends the control of a list of 132 substances in industrial effluents. If organic compounds are most of the time listed with their full chemical formulation, recommendations towards metal species are either very vague ('Arsenic and its inorganic forms, mercury and its organic derivatives, cadmium and its compounds,...') or are too accurate and lists the full formulation with the counterion. This is the case for organotin compounds. This accuracy is misplaced since most metal species determination methods use a derivatization stage for gas chromatography separation methods and therefore use the substitution of the counterion. Very little routine laboratories can offer the full service for the complete control of the 132 substances in the industrials effluents.

The present status of instrumental development of both separative and detection methods is not an obstacle anymore to the routine determination of organometallic compounds in a wide variety of matrices.

21.2 FLOW INJECTION ANALYSIS

Flow injection analysis (FIA) has not been widely applied to the determination of organometallic compounds. Flow injection methods have more often been applied to the differentiation of redox species such as Cr^{III}/Cr^{VI}, Fe^{II}/Fe^{III}, As^{III}/As^{V} and Se^{IV}/Se^{VI}. Speciation using this technique should primarily be understood as a method for differential determination using on-line discriminating chemical reactions rather than as a method based on true chromatographic separation properties. However, there is much value in considering FIA introduction techniques hyphenated to atomic spectrometry. Because of the potential for performing on-line preconcentration and separation reactions, and the fact that these techniques can be very simply interfaced with a wide range of atomic detectors

such as atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS), inductively coupled plasma/atomic emission spectrometry (ICP/AES) or inductively coupled plasma/mass spectrometry ICP/MS [3]. Straight interfacing between the FIA device and the spectrometer, through standard nebulization devices, can easily be achieved. Also, the use of a gas liquid separator after on-line hydride generation reactions (or reduction to the metallic state, as for Hg) allows direct introduction of the sample, in a gaseous state, into the atomic detector. Detectors adapted to flow injection techniques in trace-metal speciation easily match the continuous flow characteristics of the technique. Flame atomic absorption spectrometry (FAAS) and inductively coupled plasma coupled to atomic emission spectrometry (ICP/AES) have been extensively used. The sensitivity required for speciation analysis is obtained by direct selective preconcentration on micro columns. The low contamination and the possibility of on-line matrix pretreatment lead to an excellent overall reproducibility (0.5–1%). Analytes can be concentrated from millilitres of sample down to few microlitres to be injected into the detector, allowing concentration factors of 50–100. Speciation conditions can be achieved using several continuous separation techniques such as dialysis, gas diffusion, ion exchange, liquid–liquid extraction or redox reactions. Despite the versatility and potential of FIA introduction methods, very few reports exist on the speciation of organometallic compounds. Here we present two very different approaches, illustrating the potential of FIA techniques when applied to the determination of organometallic compounds. Speciation between inorganic and methylmercury can be obtained using the differential affinity of sulfydryl cotton to methylmercury under various pH conditions according to the following reaction [4]:

$$\text{Fibre–SH} + \text{CH}_3\text{Hg}^+ \leftrightarrow \text{Fibre–SCH}_3\text{Hg} + \text{H}^+ \tag{1}$$
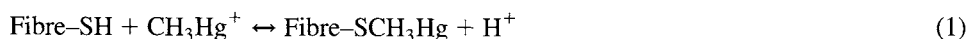
A mixed solution of inorganic and methyl-mercury is flushed onto a microcolumn packed with sulfydryl cotton in the manifold presented in Fig. 21.2. Inorganic mercury is not retained and is reduced to elemental Hg° by a $SnCl_2$ solution.

After recording the inorganic mercury peak, the elution of methylmercury occurs upon acidification of the microcolumn. On-line bromination and further reaction of methylmercury with $SnCl_2$ generates Hg° which will be detected in the atomic fluorescence apparatus. The sulfydryl microcolumns have been adapted for selective field-preconcentration of methylmercury in natural river waters at the sub-µg/l level [4].

The speciation of tetraalkylleads (tetramethyllead (TML) and tetraethyllead (TEL)) using an FIA manifold has also been reported [5]. In this case, the speciation approach is different. It does not rely on the selective retention properties of an adsorbent material as described above but is based on the differential atomization efficiencies of the TML and TEL in the flame of an AAS. The FIA manifold is interfaced to an atomic absorption spectrometer with a fuel-lean acetylene flame. Since TML gives higher absorbance peaks than TEL for a given sample, the experimental absorbance peak recorded can then be related to the respective concentrations of TML and TEL according to the following equations [5]:

$$h_t = (h_1 + h_2) = (a_1 + a_2)/2 + (b_1 \times C_{\text{TEL}}) + (b_2 + C_{\text{TML}}) \tag{2}$$

$$C_T = C_{\text{TEL}} + C_{\text{TML}} \tag{3}$$

where h_t, is the measured experimental peak height, h_1 and h_2 are the values corresponding
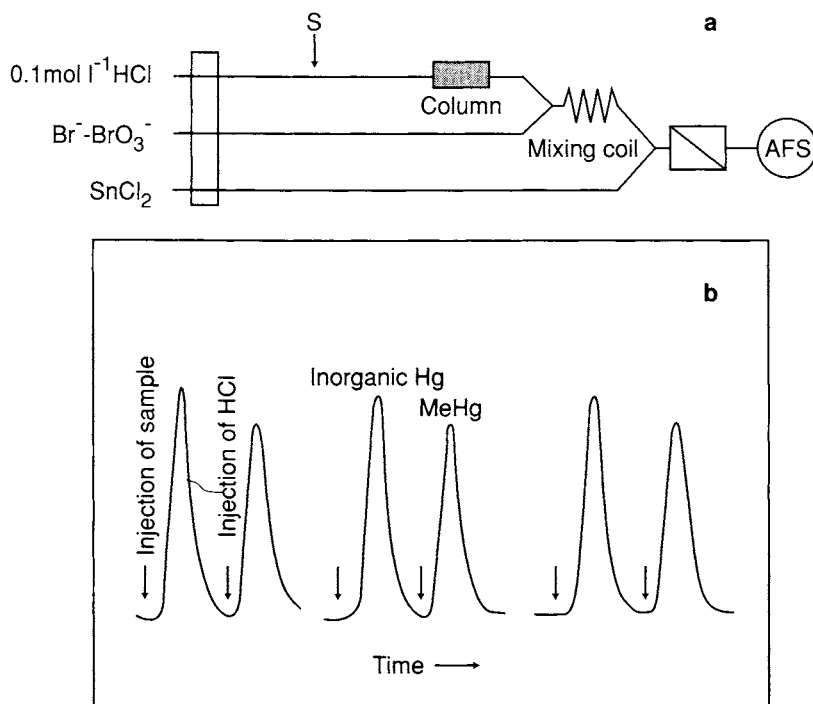
Fig. 21.2. Speciation of Hg by flow injection and detection by atomic fluorescence (FI–AFS). (a) Manifold design, MeHg in comparison with inorganic Hg is retained on the sulfydryl cotton micro-column and undergoes desorption and additional postchemical reactions before AFS detection (after [4]). (b) Reproducibility of FI–AFS, $Hg^{II}/MeHg^+$ solution 2 ng/l (courtesy of C.W. McLeod and W. Jian, Sheffield Polytechnic, UK, presented at the 1990 Winter Conference on Plasma Spectrochemistry, St. Petersburg, USA, January 1990).

to each compound (not obtained separately), a_1 and a_2 refer to the intercepts of the calibration curve from TML and TEL, respectively, and b_1 and b_2 to the corresponding slope values. The total lead value, C_T is obtained separately on the same manifold, using an on line demetallation of the tetraalkylleads by a solution of iodine in petroleum spirit (6% w/v) [5]. Limits of detection obtained for total Pb determinations are 43 µg/ml and 0.6–0.8 µg/ml for TML and TEL, respectively. The future of FIA for the speciation of organo-metallic compounds can be predicted. If manifolds and detectors can easily be assembled for this purpose, the most promising steps will certainly arise with FIA being used as an 'on-line' sample preparation procedure prior to the necessary steps to perform the specia-tion of the organometallic moieties. As an example, there is the possibility of coupling flow injection (FI) to high-performance liquid chromatography (HPLC) for on line sample clean-up, precolumn derivatization, and the elimination of matrix effects. Possibilities of interfacing FI to hydride generation/cryogenic trapping/gas chromatography/quartz furnace atomic absorption spectrometry (H/CT/GC/QFAAS) also exist, and need to be explored. In general, the automation of FI-hyphenated systems will allow higher sample through-puts. Further, if species could be preconcentrated from the sample, in the field,

under good conditions, with stable storage on the same solid support, and later eluted directly into a FI-hyphenated system, this could change the prospects of environmental speciation analysis, reduce sample sizes and the analytical errors associated with sample contamination through handling.

21.3 VOLATILIZATION REACTIONS, CRYOGENIC TRAPPING, CHROMATOGRAPHIC SEPARATION AND ATOMIC SPECTROMETRY DETECTION

Similar to flow injection methods, the technique combining on-line hydride genera-tion, cryofocusing and chromatographic separation prior to detection by atomic spectro-metry has received considerable interest during the last 15 years. This hyphenated technique was first used in 1975 for the determination of methylated forms of selenium in freshwater environments [6]. Since then, it has been used for the speciation of arsenic, antimony, selenium, germanium and recently mercury. In recent years, it has been extensively applied to the determination of organotin, and more specifically butyltin compounds, in the environment because of its simplicity and high sensitivity. The on-line integration of the different steps gives the technique a wide range of applications. All types of samples have been approached with this technique: air, water, sediments and biological tissues.

In comparison with standard hydride generation methods, the introduction of the cryo-genic and chromatographic separation steps provides both the high sensitivity and specia-tion ability required for environmental applications. In addition to the direct gain in sensitivity achieved by hydritization and cold trapping for elements such as As, Bi, Sb, Se, Sn, Ge and Te, the system provides the potential for redox speciation of inorganic species of As (III and V), Sb (III and V) and Se (IV and VI). It is also very efficient for most low boiling point alkylated species of environmental significance [7]. Organogerma-nium compounds, such as the mono-, di- and trimethylated forms, have been reported to occur in oceans [8]. Arsenic and selenium species frequently detected by this technique include monomethylarsonate (MMA), dimethylarsinate (DMA) species and dimethylse-lenide, dimethyldiselenide and diethylselenide. Both these elements have higher organo-metallic forms that are not directly amenable to gaseous derivatization methods. Species such as arsenobetaine, arsenocholine, arsenosugars for arseno- or selenomethionine or selenocysteine, require a wet digestion and determination as the inorganic form. HPLC combination methods are better suited for the direct species separation and are described later in this chapter. Applications of cryogenic systems have gained increasing popularity for the determination of organotin compounds. Methyl-, ethyl-, and especially butyltin (including tetrabutyltin) species have received considerable attention in the last few years in a wide range of samples [9]. Finally, mercury (inorganic and methyl-, dimethyl-, diethylmercury) and lead (inorganic and methyl- and ethyllead) species can be detected with a slightly modified version of the instrumentation [10,11].

21.3.1 System design

This technique combines four basic stages: on-line aqueous derivatization of the analytes; preconcentration by cryofocusing; chromatographic separation; detection by

Fig. 21.3. Hyphenated system for the speciation of As, Sn, Hg, Se and Pb developed at the University of Bordeaux I. (a) Reaction flask; (b) three-way valve; (c) trapping column packed with chromatographic material and heating wire; (d) removable liquid N_2 Dewar; (e) quartz furnace atomizer; (f) atomic absorption apparatus.

atomic spectrometry. The most frequently used detectors are atomic absorption (AAS), atomic fluorescence (AFS) in the particular case of mercury, or sometimes mass spectrometry (MS). The success of the technique lies in the compactness of the system design, the integration on-line of the different analytical steps, and the potential for addition of prior sample pretreatment procedures. Details of the analytical system are presented in Fig. 21.3. Hyphenation between the different analytical stages is very simple, since after the volatilization the analytes are processed and introduced into the detector in the gaseous state. However, a careful design of the system is necessary to minimize the dead volume and to give optimum working conditions. The reaction vessel for the derivatization reaction is usually constructed in borosilicate glass. The volume is highly variable and can range from a few millilitres to 500 ml, according to the type of species and sample investigated. The reaction flask should be

shaped so that entrapment of volatile species can be avoided [12,13]. The bubbler can either enter the sample or lie slightly above it (foaming samples) [9]. Passivation of the glass surfaces was only found to be necessary for organogermanium determinations [8,14]. Recent improvements in the hydride generator design have been proposed. The reactor is composed of a Buchner funnel and an outer glass cylinder, and the reaction takes place in the funnel where the sample and reagents are mixed. The carrier gas flows continuously through the fritted disc and sweeps the hydrides to the detectors. This simple, continuous hydride generator combines hydride production and gas-liquid separation steps, leading to an improved signal to noise ratio and efficiency in stripping the hydrides from the solution [15]. Removal of the water generated during the derivatization reactions may be necessary and improve the reproducibility in performance of the instrumentation. Solid drying agents are to be avoided since they will also irreversibly trap some of the analytes. Cryofocusing of the analytes is performed in a small (30–45 cm long, 6 mm i.d.) packed chromatographic column immersed in liquid nitrogen ($-196°C$). This cryofocusing trap is also used as a limited chromatographic separation column after it is removed and heated by a Nichrome wire. Despite its low resolution ability (1300 theoretical plates [9]), the quality of the packing phase is of prime importance for the reproducibility and peak resolution. However, such a simple chromatographic step is quite sufficient to separate most organometallic compounds of interest (one to eight species can be present on a single chromatogram). However, such a simple chromatographic step is quite sufficient to separate most organometallic compounds of interest (one to eight species can be present on a single chromatogram). The quality of the chromatography support, mesh size, and quality of the stationary phase are critical. Mesh sizes of 80–120, with 10–20% loading of non-polar stationary phases (silicone-based, such as OV 1, OV 3, OV 101, SE 50, SE 54, and SP 2100) have been used for the determination of methylated tin, alkylleads and methylated mercury species [10,15–21]. Lighter loadings (3–5%) are necessary for methylated arsenic [22] or selenium species [23] and higher boiling points species such as butyltin compounds [24]. In some systems, a preconcentration stage prior to the chromatographic separation has been reported. Trapping of volatile organotin compounds at room temperature can be performed on Tenax GC for preconcentration purposes [25,26]. Derivatized ethylated mercury species can be trapped and separated on Carbotrap columns [27].

   The on-line preconcentration and separation of derivatized species is one of the major advantages of the H/CT/GC/QFAAS method. Cryofocusing allows preconcentration factors of 50–100-fold. Separation is achieved upon warming of the trap and species are eluted on the basis of their boiling points and according to their chromatographic proper ties. The combination of this technique with the highly specific detection by atomic absorption spectrometry using deuterium background correction allows selective recording of inorganic and organometallic forms of the metals present in the samples on the same chromatogram (Fig. 21.4). In general, the reproducibility with such systems ranges between 5 and 15% ($n = 5$). Careful automation of each analytical stage should considerably reduce the relative standard deviations obtained routinely. A minimal dead volume is essential for high sensitivity and low tailing of the chromatographic peaks. When the system is fully optimized, absolute detection limits of some 10–100 pg of metal can be obtained for each organometallic species analyzed [16]. The combination of these low
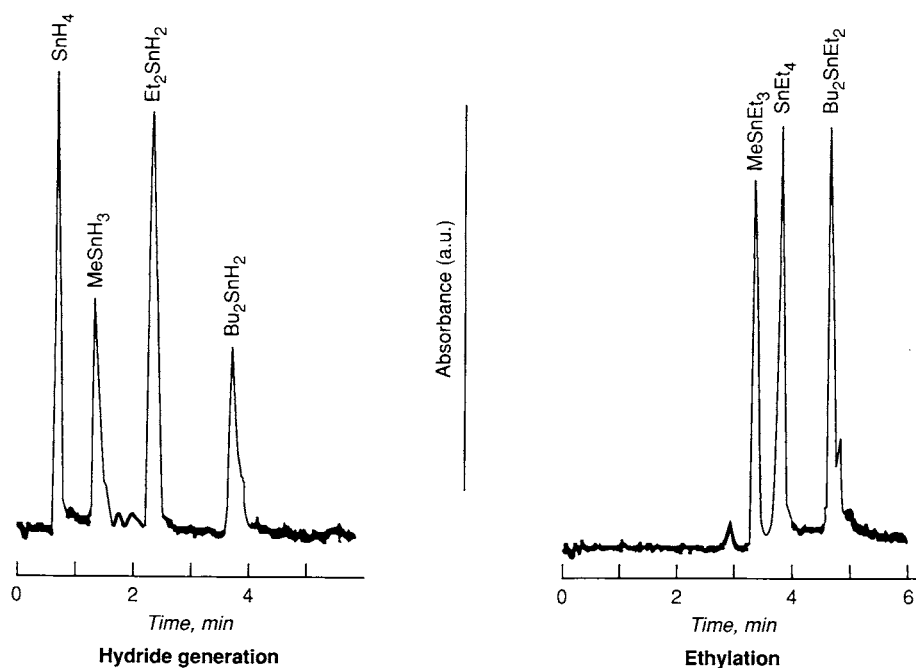
Fig. 21.4. Chromatograms of organotin compounds (5 ng as Sn in a 100-ml reaction flask) after derivatization by NaBH$_4$ or NaBEt$_4$, cryogenic trapping, gas chromatography separation and detection by atomic absorption (F.M. Martin and O.F.X. Donard, unpublished results).

detection limits with the possibility of using large reaction vessels (100–500 ml) allows the direct determination of traces of organometallic compounds in environmental waters at the ng/l level.

21.3.2 Derivatization reactions

The first step of the technique is the extraction of the analytes from the sample matrix by volatilization reactions under acidic conditions. This step is of prime importance as it serves many purposes. In addition to removing the analytes from the matrix, it also allows the on-line preconcentration which is fundamental for obtaining the appropriate sensitivity for environmental analysis. Also, if the derivatization reaction is performed under good conditions, it will minimize the elements trapped on the column and thus simplify the potential matrix interferences at the later detection stage. Hydride generation has long been the most popular method for converting the analytes into the gaseous state. Hydride generation methods have been extensively used for the determination of the inorganic species of As^{III} and As^{V}, Sb^{III} and Sb^{V} [20] and Se^{IV} and Se^{VI} [23] by selecting appropriate pH ranges for reaction. Inorganic tin species (Sn^{II} and Sn^{IV}) cannot yet be differentiated by these approaches. Hydride generation has also proved to be very efficient for the quantitative conversion of low organometallic forms of Ge, Se, Sb, As and Sn [7]. The pH conditions for reaction are critical for a quantitative conversion of the analytes (Table 21.1). In general, comparison of the pH and the pK_a of the species

TABLE 21.1

DERIVATIZING CONDITIONS FOR THE SYSTEMS USING CROGENIC/CHROMATOGRAPHY SEPARATION PRINCIPLES

Species	Reagent	Derivatization conditions	Sample pretreatment	Ref.
TRISn[a] $Me_xSn^{(4-x)+}$ $Et_xSn^{(4-x)+}$ $n\text{-}Bu_xSn^{(4-x)+}$	$NaBH_4$	1 ml of 4% aqueous $NaBH_4$	1 ml of 2 M acetic acid	[18]
TRISn[a] $Me_xSn^{(4-x)+}$	$NaBH_4$	2 × 1 ml of 1% aqueous $NaBH_4$	pH 6.5 with 4 ml of 2 M Tris–HCl	[19]
TRISn[a] $Me_xSn^{(4-x)+}$ $Me_xSn^{(4-x)+}$	$NaBH_4$	1 ml of 4% $NaBH_4$ in 0.02 M NaOH	pH 2 with 0.2 ml of 5 M HNO_3	[20]
TRISn[a] $Me_xSn^{(4-x)+}$ $n\text{-}Bu_xSn^{(4-x)+}$	$NaBH_4$	2 × 1.5 ml of 4% aqueous $NaBH_4$	pH 2 with 0.2 ml of 5 M HNO_3	[17]
$n\text{-}Bu_xSn^{(4-x)+}$	$NaBH_4$	2 × 2.5 ml of 6% aqueous $NaBH_4$	pH 1.6 with 2 ml of 5 M HNO_3	[24]
Ge Et_3Sn^+ $Me_xGe^{(4-x)+}$	$NaBH_4$	6 ml of 20% $NaBH_4$ in 0.06 M NaOH in 100 ml sample	5 ml of 1.9 M Tris-HCl + 10 ml of 300 g/l NaCl + 1 ml of 0.2 M EDTA per 100 ml sample	[8]
As^{III}	$NaBH_4$	2 ml of 2% aqueous $NaBH_4$	1–3 ml of 5% potassium biphthalate, pH=3.5–4	[21]
As^V MMA DMA Trimethylarsine	$NaBH_4$	4×2 ml of 2% aqueous $NaBH_4$	pH 1–1.5 with 5 ml of saturated solution (10% w/v) of oxalic acid in water	
$Me_xPb^{(4-x)+}$	$NaBEt_4$	3 ml of 0.43% $NaBEt_4$ in water	PH 4.1	[10]
$MeHg^+$	$NaBEt_4$	50 μl of 1% $NaBEt_4$ in water	pH 4.9 with an acetate buffer solution	[27]
Hg^{2+}	$NaBH_4$	1 ml of 0.4% $NaBH_4$ in water	pH 4	[31]
$MeHg^+$	$LiB(C_2H_5)_3H$	0.1% solution of $LiB(C_2H_5)_3H$ in THF	pH 4	[31]

[a] TRISn, total recoverable inorganic tin.

shows that the reduction is generally performed at a pH a few units below the pK_a of the species of interest [28]. The general reaction can be described for alkylated tin compounds [17]:

$$R_xSn^{(4-x)+} \xrightarrow{NaBH_4, H^+} R_xSnH_{(4-x)} + H_2 \qquad (4)$$

with $x = 1, 2, 3$ and R being methyl, ethyl or butyl.

Hydride generation was not considered to be suitable for environmentally important

methylated forms of Hg and Pb. The field of application of the D/CT/GC/AAS (derivatization/...) method has now been extended to Hg and Pb, as well as to their alkylated derivatives, by the use of a boron alkylating reagent NaBEt$_4$ [10,29]. Thus Hg and Pb can be quantitatively ethylated at pH 5 in water according to the following reactions:

$$Pb^{2+} + 2NaBEt_4 \rightarrow Et_2Pb^{II} + \cdots \tag{5}$$

$$2Et_2Pb^{II} \rightarrow Et_4Pb^{IV} + Pb^{\circ} \tag{6}$$

$$Me_2Pb^{2+} + NaBEt_4 \rightarrow Me_2Et_2Pb + \cdots \tag{7}$$

$$Hg^{2+} + 2NaBEt_4 \rightarrow Et_2Hg + \cdots \tag{8}$$

The application of this new derivatization method is under current development. It has been shown to be less sensitive to interferences than hydride generation for the determination of butyltin compounds in complex sediment matrices after an ethanolic extraction step [30]. The role of the derivatizing reaction is of prime importance. This is true for all techniques reviewed in this chapter, but here it facilitates the integration of many analytical steps required for the speciation of organometallic compounds. Further, derivatizing reactions which produce gaseous analytes from the aqueous solution also selectively limit the number of other species which could possibly interfere at the later detection stage. Finding new derivatizing reagents to achieve volatilization of the analytes will most probably be important in improving techniques which use cryogenic trapping for separations. Recently, NaBH$_4$ and LiB(C$_2$H$_5$)$_3$H have been reported to convert mercury(II) and methylmercury(II) quantitatively into volatile forms as the hydride [31,32].

Despite of its low resolution capabilities, cryofocusing brings a wide array of analytical solutions [33]. On-line volatilization and trapping allow improved separation of the analytes from the matrix, direct preconcentration and later 100% introduction in the detector. This approach yields in general excellent sensitivity and allow many atomic detectors to reach the routine sensitivity required for environmental routine analysis [33]. Mercury speciation is a critical concern for environmental issues. The development of fast and reliable sample preparation methods from both sediments and biological tissues using microwave assisted techniques has considerably simplified one of the major difficulty of the whole analytical procedure [34]. Automation of cryofocusing systems coupled to atomic detectors such as atomic absorption using a quartz furnace [34] or using atomic fluorescence should allow in the near future the commercial introduction of these analytical systems in routine control laboratories.

The ruggedness of such system when coupled to an atomic fluorescence detector has been successfully demonstrated for the field determination of volatile selenium species [35]. The recent adaptation of such system to ICP/MS allow unrivalled sensitivity for environmental routine detection [36]. Further, the capabilities of simultaneous elemental detection and further isotopic patterns allow unrivalled selectivity. Such approaches using cryofocusing allows to open new horizons since sample collection and sample determination may be easily separated. Volatile metal species can be cryogenically collected on the field and later flash desorbed in a cryofocused system in front of an atomic detector. It allows to introduce the routine monitoring of volatile metal species in industrial processes,

develop industrial hygiene aspects, and with appropriate sample preparation procedures address very low levels of metals species amenable to derivatization reactions opening the door to improved understanding of new environmental reactional pathways.

21.3.3 Atomization reactions and detection

The continuous gaseous effluent coming from the chromatographic column needs to be readily atomized in the beam of an atomic spectrophotometer. A flame, graphite furnace, or electrothermally heated cell are most frequently used for detection. The 'central problems in spectrochemical analysis are associated with atomization processes' according to Sir A. Walsh [37]. This part of the detection mode, usually performed with an atomic absorption spectrometer, is indeed critical to yield optimum sensitivity. With most hyphenated systems, the molecular identity of the analytes must be preserved for their separation by chromatographic procedures. However, since most techniques are coupled to atomic spectrometry, to obtain good selectivity and sensitivity, the alkylated species injected into the detectors must be efficiently dissociated to produce a high density of atoms in the detection cell of the spectrometer. The details and discussion presented in this section are directly relevant to speciation systems using derivatization, cryogenic trapping and separation as a separation unit, but are also directly applicable to systems based on gas chromatography or liquid chromatography followed by on-line volatilization, as described later in this chapter. At first, the interfacing between a gas chromatograph and an atomic absorption spectrometer used direct injection of the gaseous analytes into the nebulizer of a flame AAS. Some of the first reports of such interfacing and atomization methods were those by Kolb et al. [38] who directed the effluent of a gas chromatograph to the nebulizer of an atomic absorption spectrometer for the determination of alkylleads in gasoline. Similar atomization procedures have been used for the determination of organosilicon compounds [39]. However, these resulted in an overall poor sensitivity, which was possibly related to poor atomization mechanisms and a short residence time of the generated atoms in the light path of the spectrometer. Further, the combined use of the nebulizer and burner led to considerable broadening of the individual chromatographic peaks. Improvements in sensitivity were obtained by using ceramic tubes suspended above the conventional flame burner, resulting in an increase in the density of the atomic cloud and in its residence time along the optical axis of the spectrometer [40].

The graphite furnace has also been seldom used as a means of atomization after GC separation [41–43]. A simple heated stainless-steel transfer line can connect the outlet of the gaseous separation device and inject its effluent directly into the graphite tube. The graphite tube can be fired sequentially, within the elution interval of the organometallic species for germanium species [8], or continuously during the chromatographic elution. These repeated firings considerably reduce the lifetime of the graphite tube which is expensive. The atomization mechanisms associated with this type of atomizer are believed to involve surface reactions originating on the graphite walls [41]. The decomposition of alkylated species occurs in this case well below the volatilization temperature of the metal. The addition of H_2 to the argon purge gas stream resulted in improved detection limits, probably by providing a source of active radical scavengers improving the overall atomization efficiency.

An efficient and inexpensive alternative to the graphite furnace atomizer uses a silica
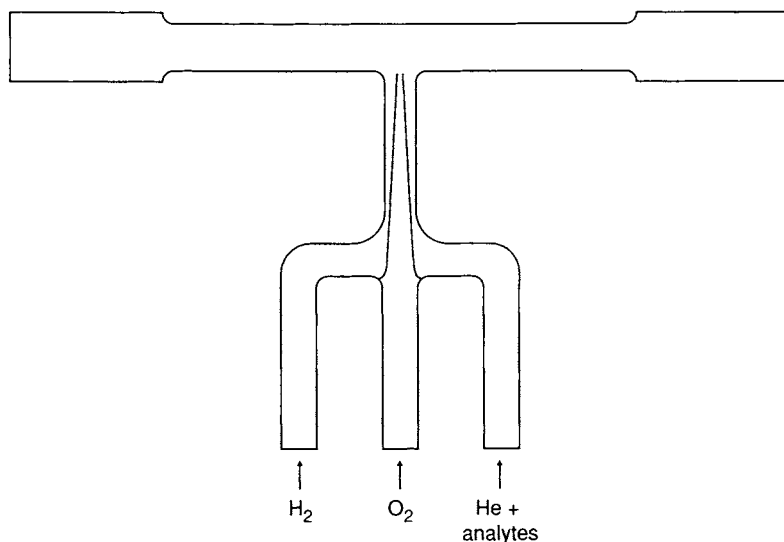
Fig. 21.5. Quartz atomizer design (light path length 19.5 cm; internal diameter 0.8 cm).

furnace, heated electrothermally. Some early uses of this atomization device were for arsine [44] and selenium species after packed column chromatography [45]. A large variety of designs has been used including one with a cell path-length of 1 m [46]. A typical configuration is presented in Fig. 21.5. Some of the important features of this atomizer are associated with the additional introduction of H_2 and O_2 gas to enhance the atomization efficiency, and the use of a narrow beam width. Both gases are reported to yield efficient atomization processes although the detailed mechanisms are not yet confirmed. Dedina and Rubeska [47] were among the first to suggest the idea of atomization of SeH_2 via free radical mechanisms and H_2 was indeed found to be essential to generate atomization. Most of the time, these quartz furnaces are operated at temperatures ranging from 700 to 950°C, which is far below that required for atomization of selenium or arsenic hydrides in graphite tubes [48,49]. It is generally accepted that atomization in the silica furnace proceeds in the presence of H_2 and O_2 via chain reactions which lead to the formation of H' or OH' radicals according to the following reactions [16]:

$$H' + O_2 \rightarrow OH' + O \tag{9}$$

$$O + H_2 \rightarrow OH' + H' \tag{10}$$

$$OH' + H_2 \rightarrow H_2O + H' \tag{11}$$

An important feature of the design of the quartz atomizer results from the necessity of delivering the mixture of H_2 and O_2 with the analytes in the carrier gas exactly at the inter section with the beam of the spectrometer. A small flame is burning and H radicals are formed either in the flame or at the beginning of the hot zone of externally heated atomizers [50].

For organometallic species, the decomposition of the molecule proceeds via a succes-

sion of electrophilic/nucleophilic reactions of the polarized hydrogenated alkylated molecule with the OH or H radicals. Atomization can be described as follows for a dialkyltin-hydride [16]:

$$R_2SnH_2 + 4H \cdot \xrightarrow{O_2,H_2} 2RH + 2H_2 + Sn° \tag{12}$$

It is important to have some knowledge of these mechanisms since interferences at the atomization stage can sometimes occur during the analysis of complex environmental matrices. These interferences can be generated when compounds co-eluting with the analytes from chromatography are present in excess over the analytes or are decomposed faster by H· radicals attack. Both processes lead to an overall depletion of radicals available for the atomization of the organometallic species and result in signal depression. In general, however, the use of a background correction lamp is not necessary, resulting only in the alteration of the signal-to-noise ratio.

The lifetime of the quartz tube atomizer is variable and can be several months. New tubes initially perform poorly and must be conditioned by several runs before giving optimum sensitivity. Contamination of the quartz surface may induce severe signal suppression in some cases. Conditioning of the furnace by treatment with 40% HF for 15 min. will improve its efficiency [51]. Devitrification of the quartz inevitably occurs and will affect both the sensitivity and precision. However, the low cost allows simple replacement. The method has now been adapted to a wide range of chromatography systems including those using capillary columns [52]. Despite the few problems mentioned above, the overall efficiency, sensitivity and low cost of the quartz electrothermal atomizer are responsible for its increasing popularity in the field of speciation of several organometallic species of Sn, Se, As, Sb, Pb and Hg.

Other detectors, such as flame photometric detectors (FPD), also provide excellent sensitivity with various chromatographic systems, but are not selective enough [20]. Finally, AFS is the preferred detector for mercury analysis. It is then more sensitive than atomic absorption spectrometry and allows picogram detection limits with the speciation of inorganic and methylated forms of mercury in the environment [27]. Applications of these techniques to the determination of As, Se, Sn and Hg in environmental samples are listed in Table 21.2.

21.4 SEPARATION BY GAS CHROMATOGRAPHY

Gas chromatography has been interfaced successfully with a wide range of detectors, since the analytes arrive at the detector in the gaseous state. Interfacing is straightforward; in most cases a simple heated stainless-steel transfer line will bring the gaseous analytes to the detectors. However, despite its apparent simplicity, this connection is critical, and needs to be mastered to achieve good separation and detection [57]. It is most important to have well-designed and well-manufactured transfer lines. They should be as short as possible and heated to avoid condensation and decomposition of the analyte. Most of the original systems used packed-column chromatography but they are now usually replaced by high-resolution capillary gas chromatography (HRCGC). HRCGC has probably indirectly increased the sensitivity of the method. Increased separation of compounds which are likely to co-elute with the organometallic species avoids competition later in the

atomization and detection processes. However, the sharp peaks obtained make heavy demands on the detector's speed of data acquisition.

The absolute detection limits obtained with these systems are similar to those using on line preconcentration since they very often use the same detector, that is atomic absorption. However, the important difference lies in the initial sample volume. With on-line hyphenated systems, the sample volumes can be as large as several hundreds of milliliters if required. Off-line hyphenated systems can only inject a few micrometers into the gas chromatograph and therefore require an important preconcentration step of the analytes prior to injection. Extraction from the sample and preconcentration then need to be performed off-line using a solvent medium, with or without chelating agents. Difficulties exist in the quantitative liquid–liquid extraction of all inorganic ionic and organometallic species. Several procedures have been developed to derivatize and concentrate the analytes after extraction from the sample, and prior to chromatographic separation. In most cases, organolead or organotin compounds react with a Grignard reagent to form volatile tetraalkyl derivatives which are suitable for chromatography.

$$R_x(Sn, Pb)^{(4-x)+} \xrightarrow{R'MgX_s/\text{solvent}} R_x(Sn, Pb)R'_{(4-x)} \tag{13}$$
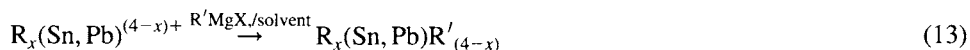
with $x = 1, 2, 3$ and R being inorganic Sn, methyl, ethyl, butyl or phenyl. For lead, R' can be propyl, butyl, or phenyl. For tin, a larger variety of R' substituents has been used, such as methyl, butyl, pentyl and hexyl. After derivatization, the excess of Grignard reagent is destroyed by the addition of sulfuric acid and the solution dried by an agent such as anhydrous $CaCl_2$. Finally, the sample is concentrated under a gentle stream of nitrogen.

These techniques have been extensively used for environmental contamination problems, with a large variety of samples (air, water, sediments and biological tissues) as shown in Table 21.2. They were principally restricted to the determination of organotin, organolead and organomercury compounds.

The widest array of analytical solutions has certainly been developed for the determination of organotin compounds; their toxicity is well established and the number of compounds under investigation is continuously rising. The protocols for alkyllead determination are very similar to those used for alkyl- or aryltins. Both hyphenated techniques most frequently rely on gas chromatography interfaced to a quartz electrothermal atomizer aligned in the beam of an atomic spectrometer, and use similar derivatization reactions. The analytical solutions used for the speciation of organomercurials are slightly different. After gas chromatography, the organomercurial compounds are usually reduced to Hg° and detected in a cold cell by AAS or by AES. This latter detection mode is the most popular for mercury after gas chromatographic separation.

The different approaches used for the speciation of important pollutants such as tin, lead, or mercury compounds are now presented. The emphasis is on the derivatization reactions and sample preparation rather than the interfacing, since hyphenation between gas chromatography and most detectors is either straightforward (as mentioned earlier) or is available commercially such as GC coupled to microwave-induced plasma/atomic emission spectroscopy (GC–MIP/AES).

21.4.1 Determination of organotin compounds

Some of the first methods concerned with the speciation and environmental aspects of

TABLE 21.2

APPLICATIONS OF HYPHENATED SYSTEMS USING CHROMATOGRAPHY COUPLED TO ATOMIC SPECTROSCOPY

Species	Detectors	Chromatography	Comments	Samples	Ref.
Applications using D/CT/GC/QFAAS					
TRISn $Me_xSn^{(4-x)+}$	QFAAS	Chromosorb GAW-DMCS 45-60, 3% SP2100	DL 20–50 pg as Sn, reagent: $NaBH_4$	Water samples, sediment, biota	[17]
$Me_xPb^{(4-x)+}$	QFAAS	Chromosorb WAW-DMCS 80-100, 10% SP2100	DL 9–10 pg as Pb, reagent: $NaBH_4$	Synthetic solutions	[10]
n-$Bu_xSn^{(4-x)+}$	QFAAS	Chromosorb GAW-DMCS 45-60, 3% SP2100	DL 11–45 pg as Sn, reagent: $NaBH_4$	Water samples, sediment	[24]
As^{III} As^V MMA DMA	QFAAS	Chromosorb WAW-DMCS 30-60, 10% OV-3	DL 200–500 pg as As, reagent: $NaBH_4$	Water samples, sediment	[52]
As^{III} As^V MMA DMA As^V MMA DMA	QFAAS	Glass beads (40 mesh)	DL 19–61 pg as As, reagent: $NaBH_4$	Water samples, pore waters	[53]
Hg^{II} $MeHg^{II}$ Me_2Hg	CVAFS	Chromosorb WAW-DMCS, 60-80, 15% OV-3	DL 0.6 pg as Hg, reagent: $NaBH_4$	Water samples, biota	[27]
$Me_xSn^{(4-x)+}$ n-$Bu_xSn^{(4-x)+}$	QFAAS	Chromosorb GAW-DMCS 45-60, 3% SP2100	DL 1.1–2.5 ng for 0.1 g of tissue (wet weight), reagent: $NaBH_4$	Oysters	[54]

TABLE 21.2 (continued)

Species	Detectors	Chromatography	Comments	Samples	Ref.
Applications using GC and various detectors					
Sn^{IV} $Me_xSn^{(4-x)+}$	QFAAS	Glass column, 6 mm i.d., 1.8 m long, 3% OV-1, Chromosorb 80-100	DL 100 pg as Sn	Water samples	[60]
Me_3SnCl Et_3SnCl Pr_3SnCl Bu_3SnCl	ECD	Glass column, 3 mm i.d., 1 m long, 20% DEGS-HG, Chromosorb 80-100	DL 1 pg as Sn	Biological materials	[93]
$n\text{-}Bu_xSn^{(4-x)+}$	FPD	CP Sil 5 CB capillary column, 25 m × 320 μm i.d, film thickness 0.4 μm	DL 0.2 pg as butyltin	Marine waters	[91]
$n\text{-}Bu_xSn^{(4-x)+}$	FPD 610 nm filter	Glass capillary column, 0.53 mm, i.d 12 m long, 3.0 μm film of BP-1	DL 1.5 ng as butyltin	Fish, sediment	[65]
$n\text{-}Bu_xSn^{(4-x)+}$ $n\text{-}Ph_2Sn^{2+}$ $n\text{-}Ph_3Sn^+$	FPD no filter	Capillary fused silica. 0.2 mm i.d., 25 m long, 0.11 μm film thickness of SE-54	DL 3–25 pg as Sn	Seawater, sediment, biota	[104]
Bu_3Sn^+ $n\text{-}Ph_3Sn^+$	ECD	Capillary fused silica. 0.2 mm i.d., 25 m long, 0.11 μm film thickness of SE-54	DL 10–300 pg as Sn	Seawater, sediment, biota	[104]
$n\text{-}Bu_2Sn^{2+}$ $n\text{-}Bu_3Sn^+$ $n\text{-}Ph_3Sn^+$	MS	Capillary fused silica. 0.2 mm i.d., 25 m long, 0.11 μm film thickness of SE-54	DL 10–60 pg as Sn	Seawater, sediment, biota	[104]
MeHgCl EtHgCl PhHgCl	ECD	FSOT column, 13 m×0.53 mm i.d., 1.2 μm film thickness of Superox- FA	DL 0.7–3.1 pg as Hg	Biological samples	[139]
MeHgCl EtHgCl PhHgCl	ECD	FSOT column RSL 300	DL 0.3–0.5 pg as Hg	Biological samples	[139]

MeHgCl EtHgCl	ECD	Packed column AT-1000	DL 4.1 pg as Hg	Biological samples	[139]
MeHg$^+$ EtHg$^+$	MIP/ AES	Packed column, 2 m × 2 mm i.d., 5% DEGS-PS on 100–120 mesh	DL 3–5 pg Hg halides	Fish tissue	[141]
MeHg$^+$ EtHg$^+$	MIP/ AES	Fused silica capillary columns, 12 m × 0.32 mm i.d., 0.25 μm non- polar DB-1	DL 0.8–1.3 pg Hg butylated	Fish tissue	[141]
MeHg$^+$	ECD and CVAAS	Packed column, 1.8 × 2 mm i.d., 5% DEGS-PS Supelcoport (no)	Not mentioned	Biological tissue	[146]
MeHg$^+$ EtHg$^+$	ECD	Capillary column, 20 m polar OV-(film thickness 0.25 μm)	DL 2 pg Hg	Fish and mussel	[140]
Me$_4$Pb Me$_3$EtPb Me$_2$Et$_2$Pb MeEt$_3$Pb Et$_4$Pb	GFAAS	Packed column, 3% OV-101 on Chromosorb W	DL 40 pg as Pb	Gasoline and exhaust air laboratory study	[55]
Me$_4$Pb Me$_3$EtPb Me$_2$Et$_2$Pb MeEt$_3$Pb Et$_4$Pb	QFAAS	Packed column, 3% OV-101 on gas chromQ	DL 12–25 pg as Pb	Rainwater, motorway run-off water, street dust aerosol	[56]
Me$_4$Pb Me$_3$EtPb Me$_2$Et$_2$Pb MeEt$_3$Pb Et$_4$Pb Me$_3$PrPb Me$_2$Pr$_2$Pb Pr$_4$Pb Me$_3$BuPb	QFAAS	Packed column, 10% OV-101 on Chromosorb W	DL 10–270 pg as Pb	Environmental samples laboratory studies	[129]

TABLE 21.2 (*continued*)

Species	Detectors	Chromatography	Comments	Samples	Ref.
Me_2Bu_2Pb					
Et_3BuPb					
Et_2Bu_2Pb					
Applications using GC/AED					
$Me_xSn^{(4-x)+}$	AED	HP-1 Capillary 25 m × 320 μm, 0.17 μm	DL 0.05 pg as Sn	Water samples, sediment	[110]
$n\text{-}Bu_xSn^{(4-x)+}$					
Pr_3Sn^+					
$n\text{-}Bu_xSn^{(4-x)+}$	AED	Capillary column, 25 m × 0.25 mm i.d. XE-52XL, film 0.25 μm	DL 6 pg as Sn	Sediment, fish	[109]

tin compounds appeared in the late 1970s and early 1980s. Meinema and his co-workers presented a method for the determination of butyltin compounds by solvent extraction, derivatization with a Grignard reagent, then gas chromatographic (GC) separation and detection by mass spectrometry [58]. Simultaneously, methods for the determination of phenyltin moieties in water, with hydridization by LiAlH$_4$, were published, using GC and an electron capture detector (ECD) or a flame ionization detector (FID) [59]. Chau and co-workers [60] then showed that atomic absorption using an electrothermal quartz furnace could be used as an efficient GC detector, which was applied to the determination of methylated tin compounds in environmental waters. Since then, a wide variety of techniques focusing on the application of gas chromatography have been published, concerning a large class of organotin species such as methyl, butyl-, ethyl-, propyl-, phenyl- and cyclohexyltin compounds in a wide variety of matrices such as natural waters, sediments and, recently, biological tissues. In general, the early papers were only concerned with the trialkylated forms, which show high toxicity. However, the demand for understanding biogeochemical pathways and degradation patterns necessitated the refinement of analytical schemes to simultaneously determine other less substituted alkyltins. The best examples of this are in papers published by Müller. His first report [61] dealt only with the determination of tributyltin in environmental samples. Three years later, his analytical methodology [62] was extended to the simultaneous determination of 19 organotin species by capillary GC and FPD.

A result of this important technical evolution is the suspicion that new organotin species occur in the environment. Mixed butylmethyltin species have been reported by several authors. Maguire [63] first reported the occurrence of such compounds in sediments. Similar findings have been mentioned by several authors [64,65]. The question that new analytical developments need to answer is whether these results represent the exact distribution of organotin compounds present in the environment or whether the species could result from the sometimes drastic conditions applied to the sample extract during determination.

21.4.1.1 Analytical scheme

Organotin analyses are difficult because with one to three substituents the compounds are polar and non-volatile, reflecting their ionic character. Quantitative methods for their determination by gas chromatography include at least seven steps. The general scheme for organotin determinations from various matrices is performed in the following order:

(a) acidification and conversion of alkyltin compounds (hydroxides, sulfides) to their respective chlorides and extraction into an organic polar solvent;
(b) purification;
(c) drying of the solvent (e.g. with CaCl$_2$);
(d) derivatization by various methods to yield volatile tetrasubstituted species suitable for GC separation;
(e) preconcentration by controlled evaporation of the solvent;
(f) separation on a GC column;
(g) detection by various types of detectors.

This analytical scheme is long and tedious to perform. The multiplicity of operations

increases the chances of errors leading to poorer reproducibility. The exact order of some steps can be changed according to different authors. Some may add a second preconcentration step which can lead to improved detection limits. The importance of the clean-up step may vary from one method to another. Finally, an important source of error arises in the preconcentration steps during evaporation. Tetrasubstituted alkyltins are sometimes highly volatile, depending on the derivatization mode used and may result in important losses during evaporation of the solvent. Nonetheless, if applied carefully this general scheme allows the efficient determination of a wide range of long-alkyl-chain organotin compounds, in various kind of matrices, as a result of the important separation capabilities introduced with capillary columns. Sample preparation procedures can be used to distinguish between the different major trends developed. They can be summarized as follows:

- derivatization of organotin compounds by a Grignard reagent;
- conversion of organotins to their hydride derivatives;
- conversion of alkyltins to the chloride salts.

21.4.1.2 Derivatization by Grignard reactions

This analytical scheme involves the reaction with a Grignard reagent, in a solvent, to convert alkyltins $(R_xSnX_{(4-x)})$ into non-polar mixed tetra-alkyltins which are suitable for GC separation. This protocol has been applied successfully to the largest number of alkyltins in different matrices, such as waters, sediments and biological tissues.

After acid digestion of the sample, the organotins are extracted into a non-polar solvent. Commonly used solvents are benzene, pentane, hexane and dichloromethane. Acidification of the sample is usually performed with HCl. The addition of HBr has been shown to enhance the recovery of organotin species [58,60,65–67], probably by preventing adsorption on the walls of the container. The first extraction scheme with a solvent is most often sufficient for trisubstituted alkyltins but highly polar mono- and di- methyl or butyltin compounds including ionic Sn^{4+} require complexation with fresh tropolone solution to extract these species efficiently into the solvent [60,62,66]. These conditions are not directly applicable to methylated tin species. More drastic acidic conditions using a combination of hydrobromic, hydrochloric, acetic and sulfuric acids in a high ionic strength medium are required [60].

Derivatization reactions have to be performed in an aprotic solvent and a drying stage is required prior to reaction. Peralkylation with a selected R' Grignard reagent (R' can be methyl, ethyl, butyl, pentyl or hexyl) in a solvent substitutes an alkyl group for the counterion of organotin compounds, to convert them into volatile tetraalkylated derivatives. The choice of the size of the alkyl group, R', depends both on the volatility of the compounds to be determined and on the type of analytical instrument used. High efficiency capillary gas chromatography allows derivatization with hexylmagnesium salts, to generate stable derivatives. The group R' can be methyl for the determination of butyltin [58,61] or phenyltin compounds [67]. Butylation has also been used for the determination of methyltin species in the environment [60]. Methylation or butylation have been abandoned, because mixed methylbutyltin species have themselves been suspected to occur in the environment. Derivatized compounds should have very different and higher boiling points than the solvent, to allow the preconcentration by evaporation. Ethylation [62] and

pentylation [65,68–71] have been successfully applied to the determination of organotin compounds in the environment. Finally, hexylmagnesium bromide was used to improve the separation between butyltin species and to minimize losses during sample concentration [72–74]. Hexylalkyltins are also more thermally stable, facilitating the later mass spectral determination. However, several precautions should be taken to ensure optimal determinations during this long analytical procedure. An internal standard is usually added at the beginning of the scheme to establish the efficiency of the protocol. One should use more than one internal standard with a large difference in boiling points if several alkyltin species are to be determined. Differential evaporation during the preconcentration steps could lead to errors in quantification [30]. Tropolone should be used in the dark, since dismutation of trimethyltin to tetramethyltin in the presence of light was reported [75].

New developments in the sample-preparation stage allow simpler and faster methods for the determination of alkyltins. Liquid–liquid extraction should soon be replaced by simultaneous liquid-solid partitioning and preconcentration. To simplify parts of the analytical protocol, the liquid–liquid extraction and preconcentration steps have been substituted by liquid-solid extraction. Müller first used an apolar resin (Bio-Beads SX-2) to remove tributyltin bromide from aqueous solutions and obtained good recoveries [61]. Organotins were later extracted as chlorides from tap water using a tropolone-C_{18} silica cartridge [62]. Important progress is to be expected with these solid extraction/preconcentration procedures in the future.

The general advantages of this analytical protocol are linked to the fact that Grignard derivatization yields stable tetrasubstitutes with low evaporative losses when using alkylating reagents with the higher alkyl- or arylgroups. Polar clean-up procedures are also less likely to simultaneously extract the non-polar derivatives [76].

21.4.1.3 Hydride generation

Derivatization by hydride generation is also widely used for the determination of alkyltins. Two general approaches have simultaneously been developed and adapted for gas chromatography-based instrumentation. The first uses extraction of analytes by direct hydride generation from an acidic digest of the sample, then trapping of the hydrides and separation by gas chromatography. Like these techniques, it is most efficient with small alkylated compounds (methyl- and butyltins). Applications have mostly focused on de termination of methyl- and butyltin compounds in water and sediment matrices. Trapping of the hydrides can either be done cryogenically [77] or at room temperature on Tenax, using a commercial purge-and-trap system [78,79]. The detectors used are either classical FPD or mass spectrometry [80]. These purge-and-trap techniques have revealed the occur-rence of volatile methyltin hydrides in the environment [81].

The second approach is also based on liquid–liquid extraction of organotin compounds from the sample, but the derivatization is then performed with borohydride in an aqueous medium or in another solvent [82–87]. The organotin hydrides are similarly concentrated by slow evaporation of the solvent and injected into a gas chromatograph for separation and detection. This procedure has mainly been applied to di- and trialkylated compounds such as the butyl- and phenyltins. It has been applied to water and sediment samples but most of the applications reported have been developed for analysis of biological tissues. All techniques include an important clean-up step which is usually accomplished by

passing the extracted compounds in the solvent (preferably hexane to prevent water deactivation of the cartridge) through a silica microcolumn. Since most of these techniques are focused towards the determination of organotin compounds in biological matrices, the products of the first extraction procedures yield high levels of lipids. Direct determination of organotins can be performed after this first extraction but the high lipid content will degrade the column, generating erratic reproducibility, and prevent good concentration of the solvent, thereby leading to poor overall detection limits. The cleanup procedure removes more than 90% of the lipid content of the sample but does not eliminate pigments or other extraneous material [86]. Analyte recoveries should be carefully checked, since in the case of Ph_3SnCl, 75.9% was obtained for a 10 μg/g concentration whereas it was only 33% for a 0.1 μg/g concentration [84]. Despite this problem, the clean-up stage has generally been shown to increase the sensitivity of the analysis, and to improve tailing of the chromatographic peaks by drastically reducing the occurrence of interfering compounds. Several types of commercial silica gel cartridges have been tried and all gave satisfactory results.

As with the analytical protocols using alkylation via Grignard reactions, many authors have tried to eliminate some steps. Extraction and preconcentration of tributyltin compounds from estuarine waters has been achieved using a bonded C_{18} solid phase adsorbent [88] prior to hydride derivatization.

The simultaneous extraction into dichloromethane and hydritization by $NaBH_4$ of butyl- and methylbutyltin species was shown to yield excellent recoveries of these compounds from natural water samples [45,64]. This method can process large sample volumes (800–1000 ml) and thus gives good detection limits, expressed on a concentration basis [89]. A new trend is also appearing in the literature. On-column hydride generation allows the direct injection of a solution of butyltin chloride into the gas chromatograph [86,90]. Derivatization of the extracts is performed directly at the top of the column either on solid $NaBH_4$ pellets introduced at the entrance of the chromatographic column [90] or via a packed reactor placed in the injection port of the gas chromatograph [86].

An alternative to the classical hydrogenation step using $NaBH_4$ in water is presented by the use of $NaBEt_4$ to form ethylated organotin species. This method was introduced for the determination of alkylleads and alkylmercury compounds by cryogenic trapping, as mentioned earlier in this chapter [10] and has been successfully applied to the determination of organotin compounds in water [91,92] (Fig. 21.6) and sediments [30]. In contrast to the hydride technique, this method does not appear to suffer from interferences present in sediment extracts.

21.4.1.4 Determination of the chloride salts

Since the electron capture detector is sensitive to both trialkyltin, dialkyltin [93–95], triphenyltins [96], and to tricyclohexyltin chlorides [97], there have been several developments which allow quantitative data to be obtained from biological material. In general, the detection limits obtained with the electron capture detector are poor. In comparison to methods using Grignard reagents, these methods are limited in their detection limits and by the range of alkyltin species determined in one sample. Despite the limitation mentioned above, some of the interest in the detection of organotins as their chloride salts relates to the possible direct concentration of trialkylated compounds, 'in the field',
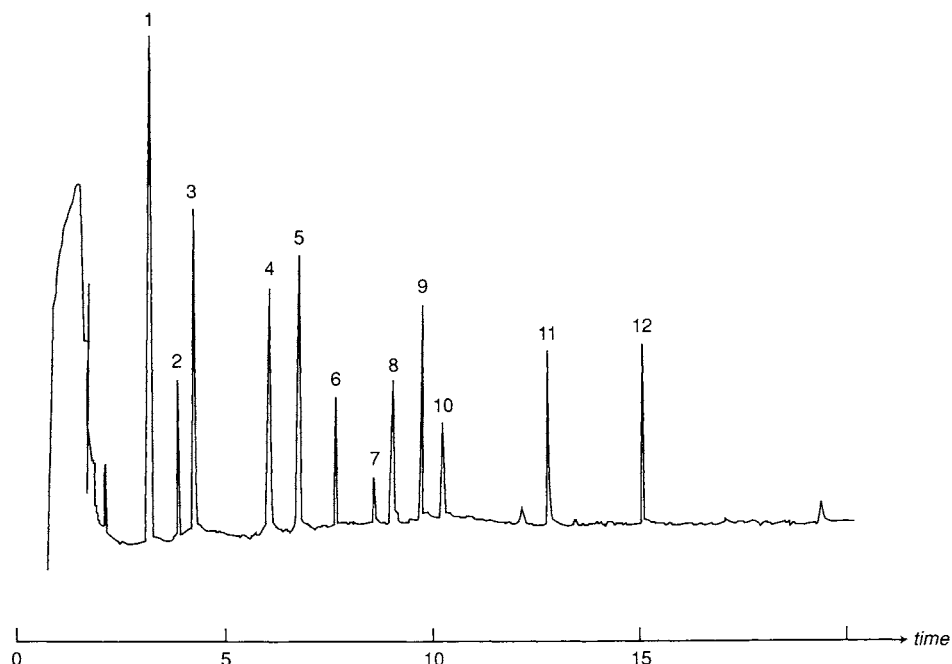
Fig. 21.6. Chromatogram of organotin compounds after simultaneous aqueous extraction derivatization by NaBEt₄ by capillary gas chromatography and flame photometric detection (initial compounds prior ethylation reaction). 1, Methyltin trichloride; 2, methylbutyltin dibromide; 3, tetra ethyltin; 4, butyltin trichloride; 5, tri-n-propyltin chloride; 6, dibutyltin dichloride; 7, phenyltin trichloride; 8, tributyltin chloride; 9, octyltin trichloride; 10, tetrabutyltin; 11, dodecyltin trichloride; 12, triphenyltin hydroxide. All compounds 50 pg as Sn; after [92].

using a solid phase adsorbent. In estuarine and marine waters, where toxicity problems are most acute, there is a high probability of the occurrence of these compounds as chloride salts. Their direct extraction by adsorption/preconcentration on a C₁₈ bonded column, then gas chromatographic separation, and detection by ECD suppresses many analytical steps in the determination protocol [98]. This also combines sample preconcentration and may facilitate storage problems. The sampling and preconcentration step can also be performed simultaneously with the use of an in situ sampler.

A time-integrating, remotely moored automatic sampling and concentration device allows direct concentration of butyltins on an octadecyl (C₁₈) bonded reversed-phase solid sorbent [99]. This approach looks promising but redistribution of the compounds has been reported. Highly acidic, non-hydrogen bridged Si–OH groups, found in the silicate sorbent phases may result in important mis-identification of the initial alkyltin species distribution in water samples.

21.4.1.5 Detectors

Several types of detector have been used for the determination of alkyltin in environmental matrices. Their technical specificities have closely controlled their extension and

possibilities and the mode of preparation of the analytes. One of the first classical GC detectors was the electron capture detector (ECD). Other popular detectors in increasing order of specificity are the flame photometric detector (FPD) and the use of atomic absorption with atomization in an electrothermal quartz furnace. Other detectors are being used, among them mass spectrometry. Environmental analyses by gas chromatography and mass spectrometric detection were developed relatively early [58] but were not very sensitive. Considerable improvements in sensitivity have been obtained by coupling cryogenic trapping before the chromatographic stage, which allows the detection of picograms of alkyltins in various matrices [80].

The ECD detector has been shown to be efficient for the detection of organotin as chlorides, but is less sensitive and specific than the other detectors mentioned previously. The flame photometric detector is certainly now the most popular for organotin determination. However, tin is difficult to excite thermally in flames and several important developments and modifications have been made to conventional FPD for alkyltin determination [92,100–103]. Quantitation is based on monitoring the red fluorescence molecular emission of the Sn–H species at 609.5 nm. This kind of detector yields excellent sensitivity [62,104]. The best overall sensitivity is obtained with a filterless FPD, but then the detector lacks specificity [104]. To improve the selectivity, an additional scan can be per formed in the broad and less sensitive 360–490 nm blue region (SnO band) [102]. Interference in the detection stage have been mentioned with various kinds of detectors. The most common interferences reported for the FPD detectors are generated by the presence of sulfur compounds, hydrocarbons and germanium species [20,77,105]. The most specific detector so far is atomic absorption with an electrothermally heated quartz furnace (QFAAS). Many couplings with gas chromatograph instruments have been mentioned. This detector is generally highly selective for tin but it can also suffer from interferences generated by the presence of hydrocarbons [106]. In general, good agreement is obtained between results obtained with detectors such as QFAAS and FPD [107]. Some references mention the use of a promising detector for the determination of organotin compounds based on the fact that in the vapor phase they have been found to quench ionization in a hydrogen-atmosphere flame [108]. When this system is used as a chromatographic detector, the organotin compounds produce negative peaks while hydrocarbon compounds produce a positive response that is 105 times less sensitive than that of the organotin. The sensitivity of this detector is equivalent to that of a classical flame ionization detector (FID) but its tremendous advantage is its selectivity against hydrocarbons [105].

Recently, other types of detector have been used for the determination of organotin compounds after CGC separation. MIP/AED for organotin is gaining increasing popularity owing to its selectivity and high sensitivity [109,110]. These techniques feature extremely low absolute detection limits, estimated as 0.05 pg of Sn [110] and will certainly contribute to our understanding of the bio-geochemical pathways of tin in the environment. Their is no doubt that the future will see hyphenation between supercritical fluid chromatography (SCF) directly interfaced with MIP/AED detectors [111]. ICP/MS detectors have also been interfaced with gas chromatography for the determination of tin compounds [112]. Suyani and co-workers [113] have interfaced a helium MIP cavity to the MS detector of ICP/MS after gas chromatography separation. These expensive solutions

allow ever-lower levels of simultaneous detection for elements and the ability to obtain isotope ratio information.

21.4.2 Organolead compound determination

Organolead compounds form another class of organometallics which have been massively introduced into the environment. Most sources originate from tetraalkylleads (R_4Pb) used in petrol additives as antiknocking agents. Their wide dispersion in the environment and subsequent degradation give rise to a wide variety of methylated, ethylated and mixed ethylmethyl-lead species. The tri- and dialkyl-lead compounds are fairly persistent species and are ubiquitous in the environment [114]. A wide array of techniques has been developed and all are very similar to those applied to organotin compounds. Alkyllead determination has certainly been attempted for the widest array of matrices, such as air, water (river, estuarine, rain and tapwater), sediments, biological tissues [115], and even snow samples [116].

21.4.2.1 Extraction and derivatization

The extraction and derivatization procedures are quite similar to those applied with organotins. The R_4Pb compounds are simply extracted directly from environmental samples by a variety of organic solvents such as hexane or benzene. Derivatization of the ionic (R_2Pb^{2+} and R_3Pb^+) species to produce volatile tetraalkylleads has been shown to be necessary for environmental analysis. Ionic alkylleads (R_2Pb^{2+} and R_3Pb^+)can be extracted and preconcentrated from environmental water samples in the presence of NaCl and NaDDTC in benzene [117], n-hexane [115] or pentane [118]. Sample extracts are then concentrated by gentle evaporation prior to derivatization by either n-butylmagnesium chloride [118–121] n-propylmagnesium chloride [119], or phenylmagnesium chloride [120]. As is observed for organotin compounds, recoveries of R_3Pb^+ are always high whereas some discrepancies may be observed with R_2Pb^{2+} 1509]. Water sample volumes usually range from 0.5 to 10 l. Smaller volumes may be used (0.1 l) when the determination is performed with a sensitive system such as HRCGC–MIP/AES [122]. Lobinski and Adams have compared two derivatization reactions applying either propylation or butylation for the speciation of alkylleads in rain and tapwater at the pg/l level. They report similar results except for finding higher detection levels of diethylated lead species when butylation is employed [122].

The R_4Pb species can be determined in sediments and biological tissues. Chau and co workers [123] extracted 2 g of homogenized fish tissues or 5 g of wet sediment with 5 ml of 0.1 M EDTA and 5 ml of hexane in capped test-tubes. The mixture was centrifuged and a 5–10 µl aliquot of the extract was injected into a GC/AAS apparatus. For the di and trialkylated species, a wide variety of chemical extraction and reagent combinations exists for the recovery of R_3Pb^+ and R_2Pb^{2+} from sediments and biological tissues. A typical sequence of operation is as follows, for a biological tissue [124]. After complete digestion of 2 g of fish tissue by 5 ml of tetramethylammonium hydroxide (TMAH), the solution is extracted with 3 ml of benzene with continuous agitation for 2 h in the presence of 2 g of NaCl and 3 ml of 0.5 M NADDTC. The phases are separated by centrifugation and 1 ml of the extract is butylated by addition of 0.2 ml of 0.9 M n-butylmagnesium chloride in

tetrahydrofuran. The mixture is then washed with 2 ml of 0.5 M sulfuric acid and the phases are separated. The final extract is dried with anhydrous sodium sulfate and 10 μl is finally injected into the GC/AAS. This procedure allowed the authors to report the simultaneous measurement of R_4Pb, R_3Pb^+ and R_2Pb^{2+} in a fish sample [124].

Other derivatization solutions have been attempted but do not appear to be appropriate for the determination of alkylleads in environmental samples. Hydride generation of lead species from human urine samples was not completely satisfactory because of the poor stability of lead hydrides and poor reproducibility [125].

21.4.2.2 Detectors

As mentioned earlier in this chapter, one of the first hyphenated systems was realized for the determination of alkylleads in petrol additives, using with a packed-column gas chromatograph directly connected to the flame burner of an AAS [38]. Graphite furnace atomizers have been used with AAS detection [115]. Other detectors such as AFS and ICP/AES have also been applied to detect the lead present in the effluents of the gas chromatograph. However, most popular solutions rely on the hyphenation between a gas chromatograph and atomization in an electrothermally heated quartz cell aligned in the beam of an AAS. Generally, the simple interface consist of a stainless-steel tube between the GC column and the quartz atomizer. In this case also, the transfer-line temperature has been shown to significantly influence the sensitivity for compounds requiring a high elution temperature [122].

The introduction of hydrogen to the atomization tube also improves significantly the performance of the instrumentation [119,126]. Excellent sensitivity is generally obtained with absolute detection limits between 40 and 90 pg (as Pb) [127]. Here again, capillary gas chromatography using microwave-induced plasma atomic emission spectrometry provides the most sensitive solution reported to date, with absolute limits of detection ranging from 0.02 to 0.1 pg as Pb [122] (Fig. 21.7).

Finally R_3Pb^+ can be separated directly by GC, with detection using either an electron capture (ECD) [128] or flame ionization detector (FID) [129], but the resulting sensitivity of the whole protocol is inadequate for environmental analysis.
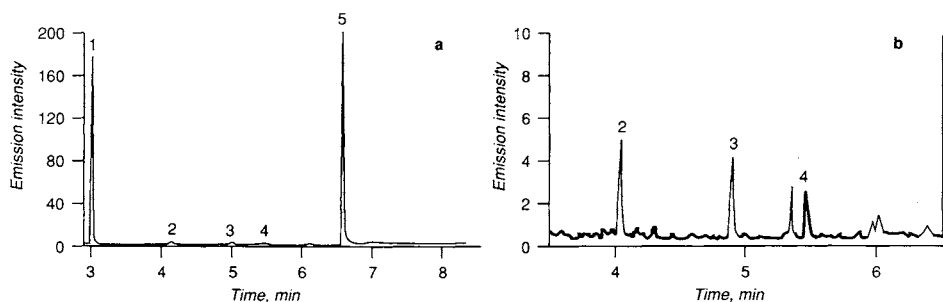
Fig. 21.7. Chromatograms of an extract of rain water sample dervatized by propylation (after preconcentration). (1) Me_3Pb^+; (2) Me_2Pb^{2+}; (3) Et_3Pb^+; (4) Et_2Pb^{2+}; (5) Pb^{2+} (reproduced from [122] with permission of Elsevier).

21.4.3 Organomercury compound determination

Mercury is certainly one of the contaminants which is best known because of the occurrence and high toxicity of methylmercury, $MeHg^+$. This is considered to be one of the most dangerous pollutants present in the environment. Concerns arise specifically since mercury accumulates in the food chain, principally as $MeHg^+$ [130]. Considerable efforts have been made to determine $MeHg^+$ principally in the environment, in air, water, sediment and fish samples. Most chromatographic applications have been focused on the de termination of $MeHg^+$ in biological tissues. However, despite the important efforts made, it is worthwhile mentioning that in comparison to other organometallic species, the method developments related to mercury speciation are not completely satisfactory and most of them are cumbersome to apply.

21.4.3.1 Sample preparation and chromatography

Traditionally, gas chromatographic analysis of organomercury compounds has been performed with packed columns and electron capture detection (ECD). The classic method for extracting and separating organomercurials from organic matrices for ECD detection is well established and commonly known as the Westöö method [131,132], focusing on the determination of $MeHg^+$. Most of the protocols used to date are derived from this methods and the general scheme can be summarized as follows:

1. Methylmercury is liberated from its protein bond by displacing the mercapto group with halogen ion at low pH
2. A selective extraction of the organomercury species is performed in an organic solvent (most frequently toluene)
3. The organic extract is purified from interfering impurities by extraction in an aqueous solution in the presence of a thiol compound (cysteine)
4. Re-dissociation of the organomercury-thiol complex, as in the initial step
5. Re-extraction into an organic solvent and eventual preconcentration by gentle evaporation of the solvent
6. Injection into a GC–ECD apparatus

A recent and slightly different version of this procedure replaces the cleaning step by repeated washing of the sample with acetone and toluene [133].

The chromatographic step of this procedure needs to be carefully controlled since the mercury-halide bond exhibits a very polar character and interacts strongly with the column, leading to severe tailing of the chromatographic peaks. To overcome this problem, 'passivation' of the column prior to determination, using a concentrated solution of mercury chloride in a solvent (benzene or toluene) has been proposed [133,134]. The chromatographic behavior and interaction of organomercury halides are still problems which are under investigation by numerous research groups. The resolution potential of capillary columns versus classical packed columns is still controversial. The first tests of wide bore thick-film fused silica open tubular (FSOT) columns (0.53 mm i.d., 1.2 μm film thickness) were reported to be very successful [135,136]. The efficiencies of high and-low polarity semi-capillary columns (SGE BP-1 (methylsilicone), BP-5 (phenyl silicone) and BP-20 (polyethyleneglycol) columns, 25 m long, 0.5 and 1 μm film) have also recently

been investigated [137]. In all cases, conditioning was necessary, but all gave satisfactory results and the use of a low polarity column for routine analysis was recommended. CP-Sil 8 CB capillary columns (wide bore and 5.35 μm thick stationary phase) were also found to be satisfactory [138]. A comparison of packed versus capillary columns (Superox-FA FSOT) has also been performed recently for the determination of alkylmercury by head space analysis using either ECD or MIP/AES detectors. The columns need to be water-resistant. In this study, there was no clear advantages in the capillary columns over traditionally packed columns [139].

In order to avoid the chromatographic problems, several alternative approaches have been used. Alkylmercurials can be converted into their bromide derivatives by the addition of cupric bromide at the clean-up stage. Determination by capillary gas chromatography (OV-275) with ECD detection yields excellent chromatographic resolution for EtHg$^+$ and MeHg$^+$ (Fig. 21.8) [140].

Finally, to eliminate the problem generated by the polar mercury-halide bond, the mercury species can be butylated by a Grignard reagent, a procedure similar to that described for Sn or Pb compounds, to yield non-polar dialkyl derivatives [141]. As the electron-capturing halide moiety is absent from these derivatives, mercury specific detection is necessary and is achieved by MIP/AES [141].
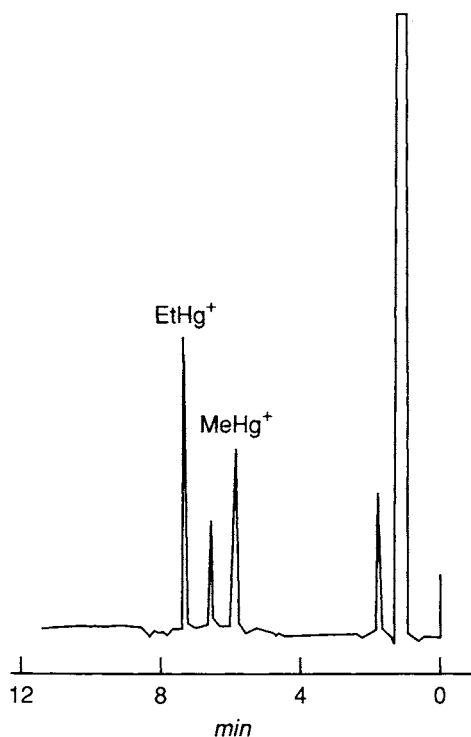
Fig. 21.8. Chromatogram from the analysis of MeHg$^+$ in an extract from an homogenized fish sample (reproduced from [140] with permission from Springer-Verlag).

21.4.3.2 Detectors

The non-specificity of the ECD detector and the possibility of co-elution with other compounds have triggered the interest in interfacing chromatography systems to Hg-specific detectors. The volatility of Hg° and the ease of thermal decomposition of alkyl-mercury compounds have allowed a wide variety of solutions which use cold vapor atomic absorption determination in a fused silica quartz cell. Various methods for the reduction of mercury have been proposed. After chromatographic separation, dialkylmercury compounds were decomposed to give Hg° after passing through a flame ionization detector and detected in small cold vapor unit as detector MAS 50 [142]. Using a simple GC/AAS interface heated to 600°C, thermal decomposition of alkylmercury species (Me_2Hg, Et_2Hg, MeHgCl and EtHgCl) was obtained in the heated capillary line just prior to entry into the AAS [143,144]. Atomization of organomercurials is achieved in the FPD and subsequent detection by AAS did not yield good sensitivity, but produced pronounced tailing of the chromatographic peaks. Significant improvements were observed when the atomization and detection were performed directly in the fused silica furnace held at 780°C using an oxygen flow and after considerable reduction of the transfer lines [145]. A recent and sensitive set-up applied to the determination of $MeHg^+$ in biological tissues, hyphenates detection using the ECD with direct confirmation of Hg in the peak detected by on-line thermal decomposition (900°C) in a quartz tube, cooling, preconcentration on gold wool and final detection of the cold vapor in an AAS [146].

Despite the different combinations which use AAS as a detector, the most frequent solution adopted for the determination of organomercurials after GC separation is certainly MIP/AES, because it avoids the predecomposition step required in the AAS detection mode. The first applications of the MIP/AES detector for mercury speciation and detection were reported in the 1970s [147–149]. It has been applied since by numerous authors to the determination of alkylmercury in water, sediments [150–153] and in biological samples [154]. Headspace analysis using GC–MIP/AES has also been reported for the determination of $MeHg^+$ in fish samples [155]. This approach has recently been improved [156,157].

However, despite the final sensitivities of the detectors, most of the above methods re quire large sample volumes, tedious solvent extraction procedures, and usually lead to the final determination of only the $MeHg^+$ species. Considerable improvements are expected in this area, as mentioned earlier in this chapter. The recent description of the feasibility of quantitative in situ aqueous ethylation of ionic mercury and methylmercury ion, followed by on-line preconcentration and detection by AFS or AAS will certainly produce a wealth of information since it allows all mercury species to be detected in the same chromatographic run (Fig. 21.9). This approach still needs significant refinement, but its simplicity and sensitivity will certainly supplant the traditional techniques which use solvent extraction-based methods and GC–AAS or GC–MIP/AES techniques.

21.4.4 Capillary gas chromatography interfaced to ICP–MS

The combination of capillary gas chromatography with ICP–MS results in a powerful tool for speciation analysis in complex environmental samples owing to the high resolving power of the capillary GC technology and the sensitive multi-elemental detection capabil-
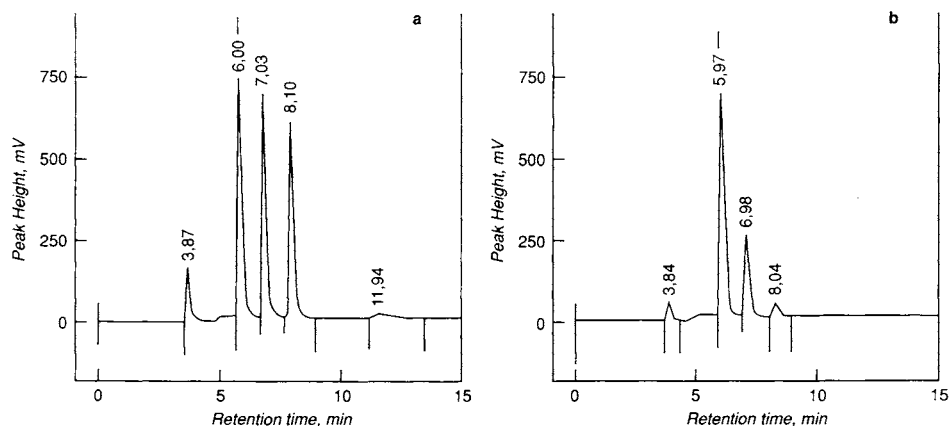
Fig. 21.9. Typical chromatograms of organomercury species obtained by aqueous phase ethylation. (a) Tap-water sample containing spikes of 454 pg Hg as $(CH_3)_2Hg$ (6.00 min peak), 429 pg as Hg CH_3HgCl (7.03 min peak; methylethylmercury) and 400 pg Hg as $HgCl_2$ (8.10 min peak; diethyl mercury). Peak at 3.87 is residual Hg°. (b) Fish tissue digestate, equivalent to about 1 mg Catostomus commersoni, spiked with 413 pg Hg as $(CH_3)_2Hg$ dimethylmercury (reproduced from [27] with permission from NRCC Canada).

ities of the ICP–MS [158–163]. One of the main advantages over GC–MIP-AES is that solvent venting (to prevent unstable plasma conditions) is not necessary in GC–ICP–MS. Interfaces between GC and ICP–MS have to be home-made, commercially available transferlines are not yet available, although at least one ICP–MS manufacturers seems to be active in developing one. Interfaces for coupling GC to MS have been tested for GC–ICP–MS interfacing for the determination of pentylated butyltin compounds [162]. In this case the GC column was fed into the ICP–MS via a T-joint (Swagelok) in order to add additional Ar (approx. 870 ml/min). This additional argon gas was heated in order to prevent condensation of the analytes. The transferline was kept at a temperature of 225°C. The last part, between the T-joint and the injector tube of the torch, was not heated. This GC–ICP–MS coupling with the (originally) GC–MS interface was not particular successful due to peak broadening. especially for compounds with higher boiling points (Bu_4Sn and Bu_3SnPe). This peak broadening was due to insufficient heating of the end of the transfer line beginning at the T-joint. Another drawback of this commercial (GC–MS) transferline was its stiff construction, resulting in frequently breaking of the column.

A more flexible transfer line was developed by de Smaele et al. [163]. Much attention was paid to overcome the problems of the commercial GC–MS transfer line. A home made developed miniaturized T-joint was designed (Fig. 21.10). The new transferline consisted of a stainless-steel tubes welded to the T-joint as shown in Fig. 21.10 (parts 4 and 5). This T-joint was placed in the oven of the GC to overcome the problem of cooling of the T-joint and to ensure isothermal conditions over the entire transfer line.

The hyphenation of the transfer line to the ICP–MS instrumentation is shown in Fig. 21.11. The transfer line consists of a electrically heated stainless-steel tube, through which an uncoated but deactivated fused silica transfer capillary is passed till the end of the ICP injector. All parts of the stainless-steel transfer tube are heated, including the part inside the torch box.
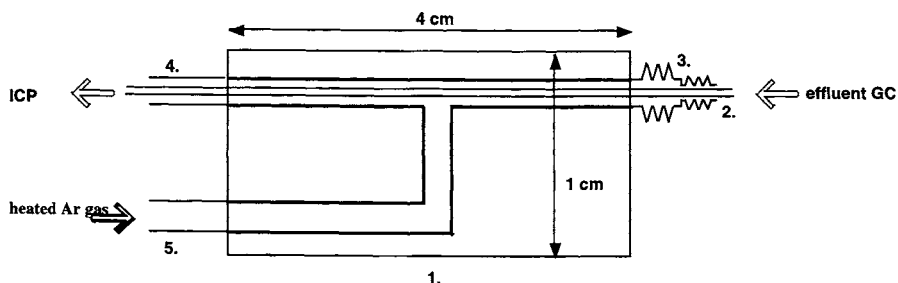
Fig. 21.10. Metal part, functioning as T-joint, to connect the two stainless-steel parts of the custom made transfer line: (1) stainless-steel piece (4 cm length × 1 cm width × 0.5 cm depth), (2) transfer capillary, (3) reducing union (1/16–1/8 inch), (4) stainless-steel tube (transfer line), (5) stainless-steel tube (Ar heater), adapted from [162].

Because ICP–MS has multi-element detection capabilities more elements can be determined simultaneously. Recently, examples of simultaneous analysis of organometallic compounds have been published [163–166]. An example is given in Fig. 21.12.

ICP–MS instrumentation is prone to signal suppressions and/or instrumental drift. These problems can be compensated by the use of internal standards. In the case of GC–ICP–MS the internal standard can be added to the carrier gas of the gas chromatograph. A suitable internal standard is Xe [163]. The ^{126}Xe signal is monitored simultaneously with the other isotopes of interest. In this way correction can be performed for instrumental drift and signal suppression.

The sensitivity of GC–ICP–MS instrumentation is excellent compared to other techniques used for speciation analysis (see Fig. 21.1) resulting in a very sensitive multi-elemental tool for speciation analysis at environmental significant levels.

If GC coupled to ICP/MS one can anticipate the advent of novel developments with the
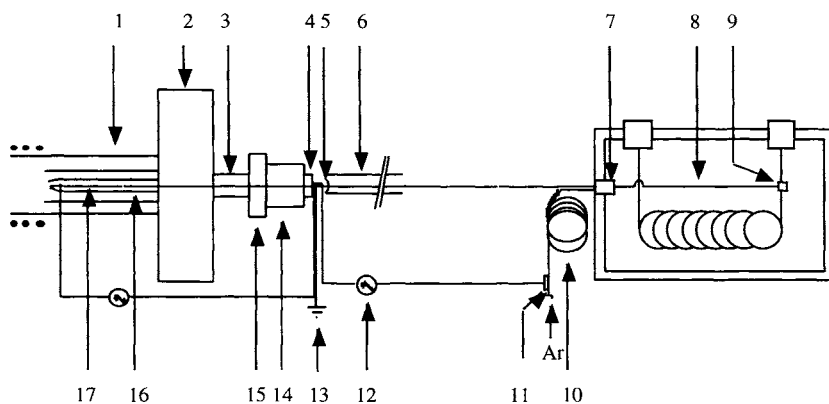
Fig. 21.11. GC–ICP–MS instrumental set-up, adapted from [163]. (1) Torch, (2) torch box, (3) injector supply, (4) stainless steel tube (transfer-line), (5) teflon adapter, (6) thermal isolation, (7) T-joint, (see Fig. 21.10), (8) transfer capillary, (9) effluent splitter, (10) Ar gas heating coil, (11) female Swagelok adapter, (12) variable AC supply, (13) earthing, (14) teflon coupling piece, (15) device attaching screw to torch, (16) stainless-steel transfer tube, and (17) transfer capillary.
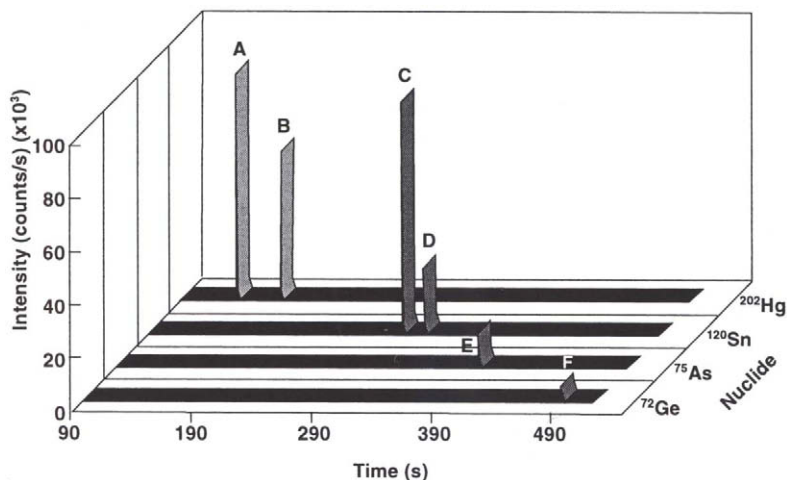
Fig. 21.12. MS chromatogram showing the separation of several organometallic compounds, adapted from [163]. Concentration range: 50–400 μg\l. Peaks: A = dimethylmercury, B = diethylmercury, C = tetrabutyltin, D = tributyl-pentyltin, E = tri(fluorophenyl)arsenic, and F = tetraethylgermanium.

introduction on different ionization sources. The plasma source provides excellent sensitivity but lead to the complete destruction of the analyte. The new development of soft ionization source using low power pressure inductively coupled plasma such as modified radio frequency glow discharge source allow the controlled fragmentation of organometallic analytes. This approach has been successfully applied to the determination of organotin compounds [167,168]. Part of the novel challenges in the future will be confronted to the identification of novel molecules containing a metal in their structure. At this stage hyphenated techniques start to merge into the field of organic analysis. If these approach could maintain the sensitivity achievements of GC/ICP/MS hyphenated system, this would then bring a major advancement in environmental sciences.

Further to improved detection capabilities, the separation aspect is gaining new horizons with the introduction of ultra-fast separation columns. Two important issues dealing with chromatographic separation and injection into the detector have been solved with the advent of multicapillary column. The introduction of this novel device in organometallic compounds determination brings several advantages over traditional gaseous separation techniques. The first important aspect is the excellent resolution capacities of this device despite of the very short time of elution. Complete separation of ethylated mercury species can be well resolved within less than 1 min [169] with excellent return to the baseline compared the several minutes traditionally obtained with traditional packed or capillary columns. Further to the increased speed of separation of the compounds of interest, the design of multicapillary column assembles a bundle of 900–2000 microcapillary columns. It also allows to use much higher flow rates than those used for traditional capillary columns minimizing the dilution factor in various type of detectors and hence resulting in improved detection limits. This device has been used with either MIP/AED or ICP/MS

detectors [170,171]. Separation can also be achieved isothermally and through traditional temperature programming.

The whole system device, column and oven are very compact and certainly open the door to novel possibilities with integration of the separation step to the detection stage in the whole analytical instrumentation, facilitating thus hyphenation and problems associated with them.

21.5 SEPARATION BY LIQUID CHROMATOGRAPHY

21.5.1 Introduction

Liquid chromatography (LC) is another popular method for the separation of organometallic species prior to detection. The use of LC, and in particular, high-performance liquid chromatography (HPLC), considerably increases the number of chemical and physical species which can be studied. Separation using HPLC offers several advantages over gas chromatography (GC). One of the major advantages is that the analytes can be separated at ambient temperature with no need for derivatization. This not only reduces the sample through-put time but also reduces possible losses during the processes. Furthermore, there are more variable operational parameters; both the stationary and mobile phase can be varied simultaneously to achieve better separation. A large variety of stationary phases is available and gel-permeation, adsorption, ion-exchange, normal- and reversed-phase chromatography allow the separation of ions, volatile species and high molecular-weight organometallics, as well as large complex biological species. The technique has better overall versatility than GC separations.

Although supercritical fluid chromatography (SFC) does not strictly fall within this category, it can be interfaced successfully to atomic spectrometric detectors. Capillary SFC is gaining popularity, especially for the separation of high molecular weight compounds, and bridges the gap between GC and LC in many respects. Although it combines the properties of GC and LC, SFC does not suffer from some of the problems inherent in the LC separation approach since the mobile phase is a gas at atmospheric pressure [172]. The column effluent is therefore compatible with many GC detectors. A possible advantage of SFC over both GC and LC is the ability to use a variety of gradients for separation. SFC has been successfully coupled to an ICP–MS detector, thereby eliminating the need for solution nebulization, which leads to poor transport efficiency into the plasma, need for desolvation and vaporization [173–176]. It displays high potential for the determination of environmentally significant levels of organometallic species.

For the separation of analytes of metallic or organometallic species, ion-exchange chromatography is the most widely applied form of HPLC because of its ability to separate free and complex ion species. Reversed-phase techniques are also widely utilized with the use of an ion-pairing agent.

In HPLC operation there is a continuous liquid flow, typically in the range of 0.05–5 ml/min of the mobile phase. The detector system must be compatible with this flow. In the flame mode, continuously operating atomic absorption spectrometry (AAS) and atomic emission spectroscopy (AES) are compatible with the effluent flow of the normal bore HPLC systems. Inductively coupled plasma (ICP) systems are also compatible with these effluent flow rates of a HPLC. The HPLC effluent can easily be introduced into the

nebulizer of a flame AAS (FAAS) or ICP instrument. An important factor in interfacing the HPLC with an AAS detector is the dispersion, not only in the column but especially in the interface tube and the AAS detector. It is clear that dispersion not only reduces the element sensitivity but can also destroy the separation originally obtained in the column.

FAAS was the first candidate for coupling with liquid chromatography but the coupling gave a low sensitivity from a weakly sensitive detector in combination with a low analyte transport efficiency (5–10%). By applying a post column derivatization, bringing the analytes in the gaseous state the sensitivity is improved greatly because the matrix is removed from the analytes of interest and furthermore, the gaseous analytes are introduced into the flame or plasma directly leading to a 100% transport efficiency. Graphite furnace (GF) AAS was the next spectrometric detection device to hyphenate because of its excellent detection power. However, the major drawback in interfacing HPLC with GFAAS lies in the incompatibility of the continuous elution of the mobile phase with the discontinuous nature of the GFAAS. For this reason it has hardly been applied for speciation analysis in the last decade and will therefore not be discussed further.

Hyphenation of LC to inductively coupled plasma (ICP) systems hardly improves the detection limits when atomic emission spectroscopy (AES) is used. However, when HPLC is coupled to inductively coupled plasma mass spectrometry (ICP–MS), a rather sensitive multi-element detection system is obtained. Many applications dealing with speciation analysis and liquid chromatography involve a ICP–MS detection system step.

The coupling of liquid chromatography with atomic spectrometry has been reviewed in several papers [172,174,175,177–188] and books [7,189–192].

21.5.2 Element selective detectors for liquid chromatography

21.5.2.1 High-performance liquid chromatography interfaced to flame atomic spectrometry (HPLC–FAAS)

The introduction of the eluate from a liquid chromatographic system into an AAS detector is not yet as well developed as for the gaseous eluate from a GC. Common flow rates in LC are typically in the range of 1–5 ml/min; the uptake rate of a flame AAS nebulizer is in the same range so it is possible to couple the two techniques directly using an interface tube. However, the main difficulty in interfacing HPLC and AAS is in balancing the flows because the optimal flow rates in HPLC are set by chromatographic separation criteria while the nebulizer uptake rate is set using the maximum sensitivity for the aspiration of standard solutions. Most flow rates for the nebulizer are higher than HPLC flow rates, so the nebulizer is starved of liquid. To overcome this problem of starvation, an additional solvent reservoir can be used at the end of the column [193], but this will lead to undesirable sample dilution. Another possibility to overcome this problem of starvation is to attach a Teflon funnel to the nebulizer [194]. Introducing a small T-piece into the transferline also prevents starvation of the nebulizer [195–197]. The effluent droplets from the column exit are caught in the funnel and nebulized one at a time. The chromatogram consists of a series of spikes but there was no loss in sensitivity because 100 μl droplets were sufficient to give a steady-state signal.

Only a few applications of the analysis of organometallics using HPLC directly coupled to FAAS have been reported in the 1980s [197–202], because of an inadequate overall sensitivity for environmental analysis. The use of the quartz tube atomizer, commonly

used in GC–AAS, has also been applied in LC–AAS after derivatization/volatilization of the analytes leading to overall better selectivity and sensitivity. This application will be discussed in one of the following section 'post-column' derivatization.

21.5.2.2 High-performance liquid chromatography interfaced to plasma emission detectors (HPLC–PED)

In comparison to hyphenation between HPLC and GFAAS or FAAS, the plasma source has the advantages of multi-element operation, easy coupling to the chromatography, responsiveness for metals and non-metals and the acceptance of the continuous flow of the HPLC eluent. Important disadvantages are associated with the sensitivity of the plasma to organic solvents and the general overall low efficiency of nebulizer systems. Three principal plasma sources which have been evaluated as liquid chromatography detectors are microwave-induced plasma (MIP), direct current plasma (DCP) and inductively coupled plasma (ICP).

The hyphenation potential of low power helium MIP is limited since the continuous HPLC flow will quench the discharge. New capillary columns with μl min^{-1} flows can be a solution to these problems in MIP interfacing, but then the sample capacity may limit any application to trace analysis.

DCP appears to offer certain advantages with regard to HPLC coupling. It appears to give a more stable plasma, especially with the introduction of mixed organic/aqueous eluents. The plasma stability will be superior to that obtained with ICP plasmas [203]. However, with DCP direct interfacing, there is the problem of high detection limits, in the 100 $\mu g/l$ range, so the environmental applicability will be limited [203]. The coupling of HPLC and DCP has been described for arsenic speciation [204]. However, the sensitivity was poor, being only 20% of that obtained with an ICP–AES.

Inductively coupled plasma (ICP) discharge was first developed in the 1960s and is now the most widely used spectrochemical source [205]. The main advantage of ICP–AES is its multi-element capability, resulting in a very short analysis time. The older instruments all have a radial view of the plasma in which the path-length is short. Detection limits are comparable to FAAS, ranging from 1 to 100 $\mu g/l$. The major drawback in the hyphenation of HPLC with ICP–AES is definitely the poor sensitivity obtained. Generally this sensitivity is not sufficient for trace element speciation. However, improved detection limits can be obtained if the path length in the plasma is increased. An axial viewed plasma will have an increased path-length, leading to a better overall sensitivity. Instruments with an axial plasma are commercially available since 1993. The newest generation of ICP–AES instruments, with the axial plasma have also improved options for background correction and spectral interference corrections Together with internal standard correction procedures, detection limits can reach similar values as obtained for GFAAS.

The low sensitivity generally reported for HPLC–ICP–AES also finds a cause in the inefficient conversion of effluents into aerosols and their transport to the plasma. Typically, only 1–5% of the sample analytes reach the plasma torch. A frequent observation has been that there is poor tolerance of the ICP for the mobile phases commonly used in HPLC, particularly with ion-pairing or size-exclusion LC separation techniques. Techniques to overcome these problems are directly related to improving nebulization rates and the transmission of the analytes to the plasma.

Different types of nebulizers can be used in interfacing HPLC to ICP. Some general characteristics are as follows [206,207]:

- The concentric nebulizer is very satisfactory for many sample solutions but tends to clog, especially with low aspiration gas flow rates. Furthermore, poor transport efficiency occurs with low gas flow rates
- The cross-flow nebulizer is similar to concentric nebulizer
- The glass-frit nebulizer is capable of handling the organic solvents commonly used in reversed-phase HPLC. Solvents with a high surface tension generate important foaming which severely affects the efficiency of the nebulization process [208]
- The Babington or V-groove nebulizer is capable of handling high salt concentrations
- The thermospray nebulizer yields 100% efficiency in aerosol production at flow rates of about 1 ml/min or more and offers optimal conditions for HPLC–ICP interfacing [207]

The nebulizers are critical for environmental applications since they directly control the overall analyte transfer to the plasma. HPLC has been interfaced directly to conventional pneumatic nebulizers of an ICP–AES, yielding detection limits in the nanogram range for arsenic compounds [209–211]. Overall sensitivity improvements can be obtained by using the direct injection nebulizer (DIN) interface [212]. With a DIN the complete sample is introduced into the plasma. However, flow rates are generally only 10% of the flow rates normally applied and are in the range of 100 μl min^{-1} leading to an overall sensitivity improvement of a factor of 3–5. Furthermore, DIN applications need good optimization and positioning of the capillary in the torchbox. High solids in the sample lead to instability of the capillary and subsequently a loss of sensitivity. For routine analysis a DIN is not recommended.

The use of thermospray sample introduction to facilitate coupling between the HPLC and ICP–AES offers substantial improvements, with reduced detection limits for most As species [211]. Up to now, the best results for interfacing HPLC with ICP have been achieved using an ultrasonic nebulizer as the sample introduction device [213]. An ultrasonic nebulizer gives an average 10-fold improvement in nebulization efficiency (i.e. 10–30%) compared to a pneumatic nebulizer (1–3%). Further, the desolvation which occurs in the nebulizer system removes most of the solvent (water and organic solvent) resulting in improved plasma torch conditions and better overall signal-to-noise ratio. In Table 21.3 the sensitivity for arsenic species for different types of nebulizers in HPLC–ICP–AES are given. It can be seen that the ultrasonic nebulization yields the highest overall sensitivity.

TABLE 21.3

DETECTION LIMITS (IN ng ABS) OBTAINED IN HPLC-ICP-AES SYSTEMS FOR ARSENIC SPECIATION APPLYING DIFFERENT SAMPLE INTRODUCTION TECHNIQUES

	As^{III}	As^{V}	MMA	DMA	PhAs	Ref.
HPLC-ICP	390	126	57	60		[210]
HPLC-DIN-ICP	19.6		11.2			[212]
HPLC-USN-ICP	6	9	3^a	3^a		[213]
HPLC-TS-ICP	234	3.4		31	2.4	[211]

^{a} Estimated.

21.5.2.3 High-performance liquid chromatography interfaced to inductively coupled plasma/mass spectrometry (HPLC–ICP–MS)

One of the limiting factors for hyphenated techniques using HPLC–PED is definitely the lack of overall sensitivity. The contrary is true for ICP–MS which has an excellent sensitivity. ICP–MS is widely used in elemental analysis because of its good sensitivity (1–50 pg abs), selectivity, the provision of generally uncomplicated mass spectra, its capability for isotope ratio determination and its multi-elemental character [174,214].

The hyphenation of LC to ICP–MS yields a very sensitive and selective analytical system for speciation analysis. The two-fold identification system LC–ICP–MS produces reliable and specific identification of the analytes in environmental applications. Detection limits in the low picogram range are within reach, and are 2–3 orders of magnitude lower than those obtained with FAAS and ICP–AES. The high sensitivity of the system also eliminates the need for a postcolumn derivatization step for most applications. However, when post-column derivatization is applied in a LC–ICP–MS system a remarkable gain in sensitivity (factor of 10–30) is obtained leading to detection limits in the picogram range, e.g. 0.6–3 pg for arsenic [215,216].

Interfacing the HPLC column and the ICP–MS detector is quite straightforward due to the compatibility of the column effluent flow from the LC and the solvent uptake requirements of most commercial nebulizers from the ICP–MS. Interfacing is achieved by connecting a short length of narrow e.g. polytetrafluoroethylene (PTFE) tubing from the column outlet to the nebulizer of the ICP–MS. However, some precautions must be addressed here. The salt content of the mobile phase must be kept to a minimum (<2% total dissolved solid) to prevent clogging of the nebulizer and erosion of the cones. Carbon build up as soot on the sampler must be prevented, limiting the use of organic mobile phases.

Sample introduction in LC–ICP–MS involves normally the standard nebulizers and spraychambers. Although, in special cases different sample introduction systems like thermospray nebulization, ultrasonic nebulizers (USN) or direct injection nebulizers (DIN) are used [217]. Similar like in ICP–AES sensitivity improvements (factor of 10–30) are obtained in ICP–MS by applying these types of nebulizers. In a recent study seven different spray chambers were tested in respect to their performance characteristics (resolution, sensitivity and signal to noise ratio) [218]. It was concluded that a cyclonic spray chamber with a cooling jacket gave a good transport efficiency (7.5%) without loss of chromatographic resolution and sensitivity.

21.5.2.4 High-performance liquid chromatography coupled to other detectors

Electrospray mass spectrometry. Electrospray mass spectrometry (ES–MS) is a relatively new technique used with success for structural characterization of numerous compounds of biological and environmental interest [219–221]. Hyphenation with microbore HPLC yields a tool for speciation analysis. When a triple quadrupole mass spectrometer is operated in the selected reaction monitoring mode a very selective tool is obtained for speciation analysis. The sensitivity for different organoarsenicals can vary largely depending of the ionization efficiency during the ES process and the intensity of the collision induced dissociation fragment ions monitored in the selected-reaction mode

[219]. Detection limits for ten organoarsenicals vary from 2 pg for arsenobetaine to 305 pg for methylarsonic acid. In relation to the low sample capacity of the microbore LC the overall sensitivity is rather poor in comparison with techniques using normal bore ICP–MS. However, the selectivity is much better for microbore LC–ES–MS–MS due to the (soft) fragmentation of the molecular ion. In this way compounds can be positively identified by its retention time of the separation column and its fragmentation patterns. The combination of LC–ICP–MS and μ-LC–ES–MS–MS can provide the appropriate tools for new elemental speciation analysis in future.

Atomic fluorescence spectrometry. Atomic fluorescence spectrometry (AFS) can be used for hydride -forming elements like e.g. arsenic [222–226]. The main advantage of AFS is the inexpensive instrumentation, the relatively clean spectra and its fairly good sensitivity. The sensitivity after hydride generation is in the low (0.05–1) ng range for LC separations which is comparable with ICP–AES and quartz furnace AAS after post column hydride generation [222–227].

Other detectors. Speciation of organometallics can finally be achieved by combining HPLC with several other detection devices such as ultraviolet (UV) absorption spectrometry, electrochemistry, laser-enhanced ionization (LEI) and laser excited atomic fluorescence spectrometry (LEAFS) [228–233]. The highest sensitivity suitable for environmental application are obtained with the last three techniques mentioned. HPLC coupled to reductive amperometric electrochemical detection resulted in good sensitivities for nine organomercury compounds[229]. Detection limits of 100–200 pg have been reported with a preliminary system that was not yet fully optimized.

Significantly higher sensitivity can be obtained by using the LEI detection mode. In this technique, two pulsed lasers are used to produce a double resonance electronic excitation state of the tin atoms present in a flame. These atoms undergo rapid collision and ionization and are quantified by the electrodes in the flame. The high sensitivity of the LEI detection mode originates from its ability to ionise virtually every atom irradiated by the laser and from the high detection efficiency. The main drawback of LEI is the occurrence of potential interferences generated from easily ionized elements [231]. This technique has been applied for the determination of alkyltins. The absolute detection limit for TBT is 60 pg and is equivalent to HPLC–ICP–MS performances (26–126 pg) [234].

Finally, hyphenation between HPLC and flame-laser-excited atomic fluorescence spectrometry (LEAFS) also allows good detection limits. For organomanganese and trialkyltin compounds detection limits of respectively 10–22 pg and 240 pg have been reported.

21.5.3 On-line postcolumn reactions

On-line post column reactions are normally applied when there is a lack of sensitivity or selectivity as is the case of LC hyphenated to AAS and ICP–AES detection systems [209,235–248]. With highly sensitive and selective detection devices like ICP–MS post column reactions are not frequently applied.

Post column reactions normally applied deal with the volatilization, generally by an hydride generation step, of the organometallic compounds to be separated. The interface which involves the use of a hydride generator for postcolumn hydride generation results in continuous, real-time signals. After HPLC separation, it converts (alkyl)metal species into their corresponding hydrides, which are then passed to e.g. a quartz tube in the AAS. The

Argon purge flow

Sample —

Buffer —

| Column | | Post column reactor | | Ice-bath | | Gas-liquid separator | | ICP-AES or AAS |

K₂S₂O₈

HCl   NaBH₄
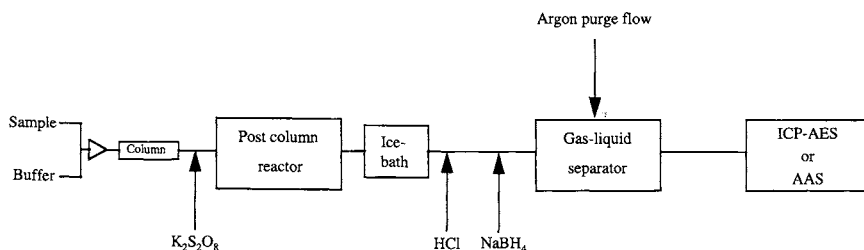
Fig. 21.13. Schematic diagram of a chemical hydride generator. The post column reactor can either be an UV photo reactor (ice-bath not essential) or a microwave oven.

hydride generation unit not only converts the analytes to volatile forms, but also isolates them from the sample stream minimizing later interferences. This technique, however, although it generates continuous signals, is limited to organometals which can form volatile hydrides. Ethylation with tetraethylborate is another possibility to generate volatile derivatives [249].

A schematic diagram of the chemical hydride generator is given in Fig. 21.13. After HPLC separation, the eluent is introduced into the hydride generator, and mixed with hydrochloric acid and then with a 1–4% $NaBH_4$ solution. The gaseous organometallics formed in the reaction coil can be separated in a gas-liquid separator and subsequently introduced, by a inert gas flow, into the spectrometric detection device (e.g. quartz-cell-FAAS or ICP–AES). Classically an all glass gas-liquid separator is used to separate the volatile species from the liquid. However, separating volatile species from the liquid is also possible by a membrane gas-liquid separator (MGLS) [215]. Two novel features were applied to reduce noise in the ICP signal: (i) addition of sweep gas in the manifold and (ii) the flow of the carrier gas through the G–L separator forms a 'feed-back' loop. In this MGLS the sweep gas, the volatile species of interest and the hydrogen migrates across the microporous membrane into the argon carrier gas, which is introduced directly into the ICP torch. By applying these two novel features noise was reduced to 50% of its original value yielding improved detection limits.

The most popular use of the postcolumn hydride generator has been for the determination of reducible arsenic species. These can first be preconcentrated onto an anion-exchange column and then eluted, by changing the mobile phase, to the separation column. In this way, improved detection limits and removal of matrix interferences can be accomplished. The analysis time can be very short; less than 10 min [195,198,250,251] or even less than 3 min [252]. With these approaches, arsenic species have been determined in a wide range of samples, such as pore waters, estuarine waters, marine animals, sediments, urine, serum, fruit and vegetables.

Methyl- and ethyltins can also be analyzed with the system presented in Fig. 21.13, after mineralization of the alkyltin compounds [199]. For methylated tin compounds, this technique was found to be superior to the H/CT/GC/QFAAS determination procedure since redistribution reactions did not take place on the chromatographic column [199]. Detection limits were of the same order of magnitude as for H/CTGC/ QFAAS; e.g. 8–14 pg as Sn. For tetramethyl- and tetraethyltin, the response was similar to that of inorganic

tin; for other compounds, the response was a function of the thermal stability and volatility of the alkyltin hydrides produced.

21.5.4 Determination of arsenic species by liquid chromatography

21.5.4.1 Introduction

The most toxic forms or arsenic found in food are the inorganic arsenite and arsenate, see also Table 21.4. Arsenic trioxide has a famous history as a poisonous compound, often used in the case of homicide. The methylated arsenic species exhibit a low toxicity while arsenobetaine is regarded to be non-toxic.

Ingestion via food or water is the main pathway of this metalloid into man, where absorption takes place in the stomach and intestines, followed by release into the bloodstream. In the liver inorganic arsenic is partially converted to a less toxic form (methylated arsenic), which is eventually excreted in the urine. Due to the differences in toxicity it will be clear that various forms of arsenic has to be determined quantitatively and qualitatively in biological fluids and tissues as well as in matrices of nutritional and environmental relevance [253]. Legal provisions are at the present almost exclusively concerned with the total amount of the element in foodstuffs and drinking water. According to the WHO the provisional total dietary intake should not exceed 2 μg of inorganic arsenic per kg of body weight.

Arsenic species of biological interest occur in the oxidation state -3 in arsine (AsH_3) with covalent bonds to hydrogen, in oxidation state $+3$ in arsenous acid (H_3AsO_3) with covalent bonds to oxygen, and in the oxidation state $+5$ in e.g. arsenic acid (H_3AsO_4) and dimethylic arsenic acid ($(CH_3)_2As(O)(OH)$ (DMA), the latter covalently bound to carbon. Arsenic is introduced into the environment from natural sources (e.g. volcanic sources and weathering of minerals) and from anthropogenic activity (ore smelting and pesticide use). Arsenic is rather mobile in the environment and can be found at concentrations of 1–2 μg/l in the world seas. Arsenate (As^V), is the predominant form found in oxic systems; arsenite, monomethyarsonic acid (MMA) and DMA also occur. The levels of arsenite (As^{III}), MMA and DMA are elevated in the photic zone of marine waters, suggesting that phytoplankton are producing these arsenic's from arsenate. Average arsenic concentrations in ocean sediments are 40 mg/kg; in coastal zones arsenic concentrations range from 3 to 15 mg/

TABLE 21.4

TOXICITY DATA OF SEVERAL ARSENIC COMPOUNDS

Compound	Abbreviation	Formula	PK_a	LD_{50}[a]
Arsenite	As^{III}	$As(OH)_2O^-$	9.3	0.014
Hydrogen arsenate	As^V	$AsO(OH)O_2^{2-}$	2.2, 7.0, 11.5	0.020
Monomethylarsonate	MMA	$CH_3AsO(O)_2^{2-}$	4.2, 8.8	1.2
Dimethylarsinate	DMA	$(CH_3)_2AsO(O)^-$	1.8, 6.1	1.8
Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$	3.6	11
Arsenobetaine	AsB	$(CH_3)_3As^+ CH_2COO^-$	2.2	>10
Arsenocholine-ion	AsC	$(CH_3)_3As^+ CH_2CH_2OH$	–	6.5
Tetrametylarsonium ion	TETRA	$(CH_3)_4As^+$	–	0.89

[a] As LD_{50} in rats (g/kg) [258,259].

kg [254]. In marine animals concentrations can be very high up to 100 mg/kg wet weight. Most of the arsenic in marine animals is present as arsenobetaine (AsB) ranging from 12 to more than 98%. Some animals contain tetramethylarsonium ion TETRA) at appreciable levels, and small quantities of dimethylarsinylribosides, trimethylarsine and arsenocholine (AsC) can also occur. The source of arsenobetaine in seafood is likely to be arsenic containing ribosides in algae [254].

Recently it was also found that arsenobetaine can also be found in the terrestrial environment [255–257].

21.5.4.2 Separation of arsenic species

The most commonly used speciation techniques for arsenic involve a combination of liquid chromatographic separation with an appropriate spectrometric detection. Two major HPLC systems, ion-exchange, and reversed-phase (RP) ion-pair chromatography, have been mainly used for the separation of arsenic compounds.

Due to the ionic character of the arsenic species at neutral pH cation-exchange and anion-exchange have been commonly used for the separation of ionic arsenic species. Reversed-phase ion-pair LC has been used with the appropriate counterions, e.g. tetra-methylammonium cation and heptanesulfonate anion in the mobile phase. The counterion forms an ion-pair with oppositely charged analyte ions and therefore additional interactions are introduced which can result in improved separations. Comparing both separation systems it can be concluded that generally ion exchange is the preferred separation system because analyte ions interact direct with the stationary phase of the column and are therefore less prone to interferences from matrix constituents other than the analyte-ion/counter-ion pair in the RP chromatographic system [220]. Furthermore, the low content of organic solvent normally used in the mobile phase of ion-exchange systems is preferable in ICP operation because higher concentrations of organic solvent can result in nonstable ICP operation. Generally the support material in ion-exchange chromatography is usually either a styrene divinyl resin or silica. Resin ion-exchangers suffer from swelling effects with aqueous mobile phases, which result in their compressibility at high pressure. Silica-based materials are mechanically stable and allow high pressure separations. However, silica bonded phase columns are chemically unstable in the high pH (>8) and low pH (<2) range.

The optimum separation system should be able to separate the most common arsenic species: As^{III}, As^{V}, MMA, DMA, trimethylarsine oxide (TMAO), AsC, AsB and TETRA in biological samples during one chromatographic run. Unfortunately these eighth species cannot be separated during one run applying one column. On an anion-exchange column the uncharged and cationic species leave the column unseparated from the solvent front. On the cationic column a similar effect is observed for the anionic and uncharged species while on a reversed-phase column only those species which have a charge opposite to the charge of the ion-pairing agent can be separated.

Chloride and chlorine containing molecules present in the sample matrix can result in interfering, $^{40}Ar^{35}Cl$ which will overlap in the quadrupole ICP–MS with ^{75}As. The chloride, which also interacts with the stationary phase, can be separated from the analytes of interest and has to elute at the end of the chromatographic run. In the case chlorinated hydrocarbons (e.g. chloroform) are used during the sample pretreatment step it is essential

that the chlorine containing solvent is removed by e.g. evaporation, before injecting the sample into the HPLC system.

With anion-exchange chromatography As^{III}, As^V, MMA and DMA can be separated from cationic, uncharged species and the $^{40}Ar^{35}Cl$ interference, see Fig. 21.14. Determination of these four arsenic species is supposed to give relevant information for evaluation of human exposure [227,260,261]. Anion-exchange separations can generally be achieved within 10 min although very fast separations (<2 min) of these arsenic species can be obtained [261].

Recently an application of a combined cation and anion-exchange column was reported [262]. By applying an anionic exchange column with a cationic exchange guard column six arsenic species (As^{III}, As^V, MMA, DMA, AsB and AsC) can be separated. Because AsC has a cationic character it was strongly retained on the guard column. AsC was eluted in a final mobile phase step with 40 mM nitric acid. In this way six arsenic species were separated within twelve min. A destabilization step of twelve min. for the anion-exchange column was needed after the elution of AsC making the total run time 24 min.

An evaluation of the potential application of a large number of binary organic acids (e.g. oxalic, malonic, succinic, malic, tartaric and maleic acid) for the separation of six arsenic species (As^{III}, As^V, MMA, DMA, AsB and AsC) on a strong anion-exchange column was given recently [263]. In this study the chromatographic behavior of six arsenic compounds was evaluated by the capacity factors k versus the pH. Tartaric acid as a mobile phase exhibits a unusual behavior for arsenous acid. There is an unexpected dependence of the capacity factor on the pH (range 2.5–7.0) with the mobile phase while As^{III} itself is a
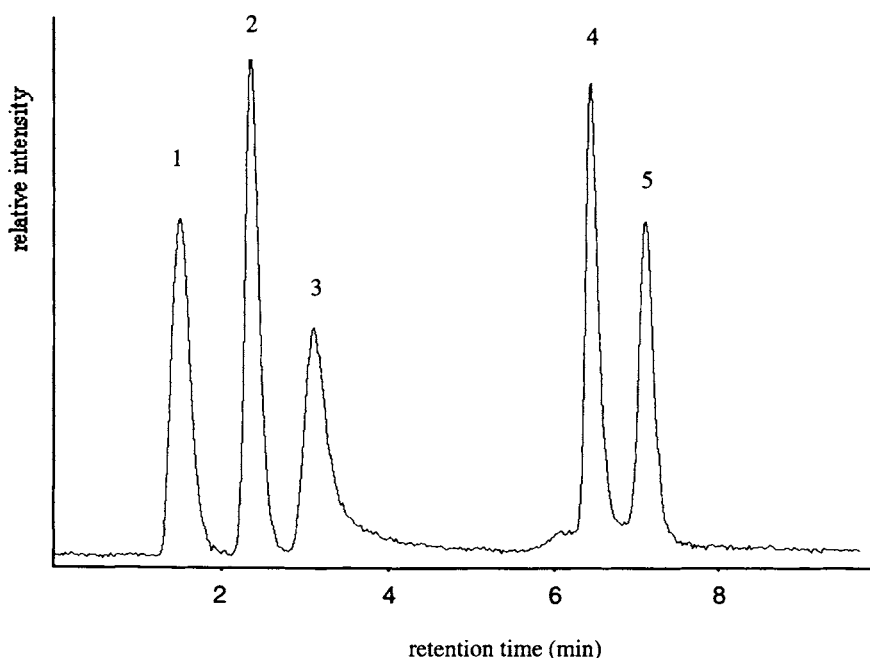
Fig. 21.14. Chromatogram of anion-exchange ICP–MS of five injected arsenic compounds (adapted from [260]). Peak identification: 1, AsB; 2, DMA; 3, As^{III}; 4, MMA; 5, As^V.

neutral molecule. A possible mechanism for this chromatographic behavior could be the complexation between arsenous acid and tartrate. Optimum separation of the six arsenic species was achieved with a 15 mM tartaric mobile phase (isocratic) at pH 2.91 within 12 min. The elution order of the six species is AsC, AsB, DMA, MMA, As^{III} and As^{V}. This elution order is very practical because there can be large differences in concentration between AsB and As^{III} in environmental and biological samples.

Only a few applications of cation-exchange chromatography have been reported [247, 258,264]. Under optimum conditions seven arsenic species (As^{III}, MMA, DMA, AsB, AsC, TMAO and TETRA) can be separated [264].

Ion-pair reversed-phase liquid chromatography is also capable of separating arsenic species [223,238,264–267]. Under optimum conditions six arsenic species (As^{III}, As^{V}, MMA, DMA, AsB and AsC) can be separated [267]. However, the elution order is such that As^{III} is eluting just after AsB which may give overlapping in the case of separating environmental or biological sample extracts.

Finally, in marine algae arsenic is mainly present in the form of relatively complex carbohydrate derivatives also known as arsenosugars. Some of these arsenosugars can be separated by gel permeation or ion-pair chromatography[268].

Finally, a wide range of applications using different stationary phases in LC is given in Table 21.5.

21.5.4.3 Detection

Direct LC–AAS and ICP–AES hyphenation is normally not sensitive enough to detect significant levels of arsenic in environmental matrices: detection limits are in the range of 100–500 ng (abs). Post column derivatization can solve this problem of low sensitivity and has been applied quite frequently [195,196,235,238–248,250–252,265,276–279]. Both inorganic arsenic species, MMA and DMA can be volatilized by post column hydride generation. Not all environmentally significant arsenic species are susceptible for hydride generation e.g. arsenobetaine, arsenocholine. These arsenic species can be volatilized by placing an 'in-line' UV photolysis coil or a microwave digestion unit between the HPLC column and the hydride generator, see Fig. 21.13. This approach allows the indirect determination of non-volatile species like arsenobetaine or arsenocholine by hydride generation. After the LC separation the column effluent is mixed with 1.5–5% peroxodisulfate and introduced in either an UV irradiated reaction coil or into a loop placed in a microwave-oven. The UV or thermo-oxidized effluent is than led through an ice-bath in order to lower its temperature, preventing over-pressure and decomposition of $NaBH_4$ in the next derivatizing step. Detection limits for AAS and AES achieved after post column derivatization vary from 0.1–1.5 ng abs.

A better sensitivity for arsenic species is obtained by AFS: detection limits: 10–100 ng (abs) depending on the sampling introduction technique applied [222,226]. However, these detection limits are still high in comparison with environmental significant levels. Again a sensitivity gain can be achieved by applying post column derivatization yielding detection limits in the range of 0.5–1 ng (abs).

Arsenic speciation has also been significantly addressed by the use of HPLC–ICP–MS [215,256,257,260,262–264,266,267,271,274,275,279–282]. Most common applied are the standard commercially available cross-flow nebulizers resulting in detection limits in the

low (10–100) picogram range Due to its monoisotopic (^{75}As) character the ICP–MS signal is excellent. Best sensitivity is obtained at high ICP power (1300 W) in combination with long dwell times (1000–2000 ms). A further sensitivity enhancement can be obtained by the introduction of carbon as methanol. Concentration between 2% and 5% (v/v) of methanol in the mobile phase can yield a sensitivity gain of a factor of 2–4, [260,267,283]. The mechanism of this carbon-mediated signal enhancement effect has been suggested as a charge transfer reaction from a large population of ionized carbon or carbon-containing polyatomic species in the plasma. The degree of ionization of the analytes like e.g. arsenic is improved by transfer of electrons to the carbon ions [283]. ICP–MS detection limits for arsenic species are far more superior than for AAS, ICP–AES or AFS and are in the low picogram range: 3–50 pg for anion exchange, 40–300 pg for cation exchange and 50–500 for ion-pair reversed phase.

Applying micro-bore HPLC–ICP–MS systems equipped with a DIN interface have also been used for As speciation, resulting in absolute detection limits of 0.6 pg As [280]. The absolute sensitivity is superior by a factor of 5–50 compared to HPLC–ICPMS systems applying conventional nebulizers. However, the major drawback is the limited sample capacity of the micro-bore column. An injection volume of maximum 0.5 μl can be introduced into the system, finally resulting in relatively poor detection limits in respect to concentration units.

21.5.4.4 Extraction and clean-up procedures for solid samples

Several methods are available for arsenic species extraction from solid materials like sediments, biological material and organisms. Methanol [265,268,270], water/methanol mixtures [242,256–258,265,269,270,284] and water/methanol/chloroform mixtures [258, 285,286] have been tested and applied. Also enzymatic digestion with trypsin can yield satisfactory results [287].

Terrestrial organisms. Arsenic species in freeze dried mushrooms have been extracted by shaking (3 times) with a methanol/water mixture (9:1 v/v) during 14 h. From the collected supernatants the methanol was removed by evaporation. Water was added to the residue and the suspension was centrifuged at 8000 rpm. After filtration (0.2 μm) of the supernatants the extract was ready for chromatographic separation [255–257].

Marine biota. Arsenic species in freeze dried mussels have been extracted (5 times) by ultrasonic agitation during 5 min at 23.5 kHz with a methanol/water mixture (75:25 v/v) [266]. From the collected supernatants the methanol was removed by evaporation. Water was added to the residue and fat was removed by a petroleum ether extraction step. If necessary, a further purification step can be introduced by applying a Waters Sep PAK cartridge (C$_{18}$ or Florisil). The final recovery obtained with this procedure was 77–95%. Before methanol extraction the lyophilized sample can be extracted with acetone to remove fat and thus avoiding interferences of fat during the clean-up step [270]. It was found that during such a defatting step with acetone only small amounts (<0.01 μg/g) of arsenobetaine were removed. Extracts are often purified when not using a highly sensitive detector like ICP–MS, where a dilution step is the best approach. General procedures make use of an ion-exchange, a C$_{18}$ column or a phenol extraction for the removal of ions and proteins and/or an extraction with a non-polar solvent for the removal of lipids [266,288,

TABLE 21.5

ARSENIC SPECIATION BY LIQUID CHROMATOGRAPHY

Species[a]	Detector	Chromatography	Comments	Sample	Ref.
1–8	HG-AFS	Anion exchange, phosphate buffer, only separation of 1–4, 100 μl inj.	$S_2O_8^{2-}$/UV oxidation, HG, 1.5% $NaBH_4$, 2 M HCl, DL 50 pg without separation	Wine, urine (2-fold dilution)	[227]
1–4	HG-AFS	Ion-pair reversed phase, tetra-alkylammonium cation, 100 μl inj.	HG, 2% $NaBH_4$, 1.5 M HCl, DL 0.4–1.2 ng	Water, SRM	[223]
1–4	HG-AAS	Ion-pair reversed phase, tetrabutylammonium cation, 100 μl inj.	HG, 2.5% $NaBH_4$, 3%. HCl, microporous membrane G-L separator, DL 0.07–0.3 ng	Groundwater, SRM	[238]
1–4	HG-AAS	Ion-pair reversed phase, TBAP gradient, 100 μl inj.	HG: 1% $NaBH_4$, 0.5 M H_2SO_4, DL 0.15–0.84 ng	Marine biota, methanol/water (1:1) extraction	[265]
1–6	HG-AAS	Anion exchange, phosphate buffer, As^{III} co-elutes with AsB, 100 μl inj.	$S_2O_8^{2-}$/microwave oven oxidation followed by HG, 3% $NaBH_4$, 3 M HCl, DL 0.8–1.5 ng	Water and urine, study of storage conditions	[241,243]
5	HG-AAS	Anion exchange, phosphate buffer, 300 μl inj.	$S_2O_8^{2-}$/microwave oven oxidation followed by HG, 2% $NaBH_4$, 3 M HCl, DL 5.5 ng	Canned seafood, methanol/water (1:1) extraction, cation exchange clean-up	[242,269]
1–4	HG-AAS	Anion exchange, phosphate buffer, 300 μl inj.	HG, 2% $NaBH_4$, 3 M HCl, DL, 0.06–0.14 ng	Serum	[244]
3–6	HG-AAS	Cation exchange, 0.1 M HCl-50 mM NaH_2PO_4, 100 μl inj.	Oxidation by $S_2O_8^{2-}$ followed by HG, 2% $NaBH_4$, 2 M HCl, DL 0.1–0.15 ng	Serum	[247]

TABLE 21.5 (*continued*)

Species[a]	Detector	Chromatography	Comments	Sample	Ref.
1–6	ICP-AES	Ion-pair reversed phase, TBAP gradient, 100 µl inj.	DL 100–130 ng	Marine biota, methanol/water (1:1) extract	[265]
5	ICP-AES	Anion exchange, phosphate buffer, 100 µl inj.	DL 10 ng	Marine biota,. Acetone defatting, methanol extraction, cation exchange clean-up	[270]
1–5	ICP-MS	Anion exchange, carbonate buffer gradient with 2% (v/v) methanol, 100 µl inj.	DL 3–4 pg	10-fold diluted urine	[260]
1, 3–8	ICP-MS	Cation exchange, HNO₃, 1 ml/min at 50°C, 50 µl inj.	DL 40–200 pg	20-fold diluted urine	[264]
1–6	ICP-MS	Anion-exchange column with cation-exchange guard column, carbonate buffer gradient, 250 µl inj.	DL 10–40 pg	Spiked river water	[262]
1–8	ICP-MS	Anion-exchange (1–4), pyridine, pH = 3.0 (formic acid), cation-exchange (5–8), Ion-pair reversed phase (2-hydroxy-5-sulfonic acid) (5–8),100 µl inj.	Hydrolic high pressure nebulization, DL 50 pg	Mushrooms, methanol/water (9:1) extraction	[256,257]
1–6	ICP-MS	Ion-pair reversed phase (tetrabutylammonium), 50 µl inj.	Addition of MeOH 2% (v/v), DL 50–150 pg	Spring and mineral waters	[267]
1–6	ICP-MS	Ion-pair reversed phase (tetrabutylammonium), 50 µl inj.	DL 120–500 pg	Marine biota, methanol/water (75:25) extraction	[266]

1–4	ICP-MS	Micellar LC, cetyltrimethyl-ammonium bromide, 100 μl inj.	DL 90–3000 pg	Urine after filtration	[271]
1, 3–5, 7	ICP-MS	Anion exchange, 10 mM tartaric acid, 20 μl inj.	DL 10–20 pg	Urine after 10-fold dilution	[272]
1–4	ICP-MS	Anion exchange, 20 mM KNO_3, optimized for inorg. As, 20 μl inj.	Also speciation of Cr^{III} and Cr^{IV} in the same run, DL 130 pg for inorg. As	Waste and drinking water	[273]
1–6	ICP-MS	Anion exchange, bicarbonate buffer gradient (38 min), 250 μl inj.	PAA internal stand, DL 10–50 pg	Fish and mussel, methanol water (1:1) extraction	[274]
1–6	ICP-MS	Anion exchange, 15 mM tartaric acid, 100μl inj.	DL 4–60 pg	Urine, water, biota	[263]
1–4,	ICP-MS,	Anion exchange, ammoniumphosphate buffer gradient, 100 μl inj.	Simultaneous separation of arsenic and selenium (IV and VI), DL 10–50 pg, Sb^V internal standard	Analytical optimization	[275]
3–8,	ES-MS-MS	Microbore reversed phase 1% acetic acid in water methanol (80–20 v/v), 1 μl inj.	Poor separation, excellent selectivity, DL 2-21 pg	Urine	[219]
1–4	ICP-MS	Anion exchange, phosphate buffer, 50 μl inj.	HG: 1% $NaBH_4$, 10% HCl, microporous membrane G-L separator, DL 0.6–3.1 pg	Sea water, RM NASS-4 and SLEW-2	[215]

[a] Species identification: 1, As^{III}; 2, As^V; 3, MMA; 4, DMA; 5, AsB; 6, AsC; 7, TMAO; 8, TETRA; HG, hydride generation; DL, detection limit.

289]. An extensive optimization of the clean-up procedure has been described recently [265].

Urine and serum samples. Arsenic speciation in urine samples is normally applied with an anion-exchange separation because the anionic arsenic species are, from a toxicological point of view the most interesting [227,241,243,244,260,264,272,277,290,291]. The inorganic arsenic in urine is partly metabolized in the human body into the methylated species MMA and DMA [260,292,293]. In urine, only As^{III} and AsC are unstable and oxidation to As^V and AsB, respectively was observed [241]. As^V, MMA, DMA and AsB are stable in urine samples for at least 67 days when stored at 4°C [241]. Urine samples can be introduced directly [290] into the LC column after a filtration step to remove precipitates or after a dilution step [227,260,272]. Freeze drying followed by a methanol/water leach has also been applied but is definitely much more laborious than the dilution/filtration steps [227]. Arsenic speciation in serum or blood is not as frequently applied as for urine samples. Only a few applications were found in literature [244,247,294,295] In one case an anion-exchange column was applied for the separation of As^{III}, $As^{V,}$ MMA and DMA [244] while a cation-exchange column was applied for the separation of MMA, DMA, AsB and AsC [247]. No special pretreatment steps for serum sample analysis were applied.

21.5.5 Determination of tin species by liquid chromatography

21.5.5.1 Introduction

Most applications dealing with the direct determination of organotin compounds in environmental samples involve a gas chromatographic separation and an appropriate elemental selective detection device. Organotin determinations on a routine base involve only gas chromatographic separations.

Few applications involving a liquid chromatographic separation have been described in the last decade. Applications for organotin determination with liquid chromatography followed by ICP–AES or AAS detection yield limits of detection in the high nanogram range and are therefor not suitable for environmental analysis. With some special care in respect to sample introduction or post column derivatization improved detection limits for ICP–AES or AAS can be achieved. Improved sensitivity (0.5 ng) was reported for real on-line LC-GFAAS employed with a thermospray interface [296].

ICP–MS detection devices have sufficiently selectivity and sensitivity (low pg range) [218,297–312]. The following sections will focus on the determination or organotin compounds by ICP–MS detection.

21.5.5.2 Separation and detection of organotin compounds

Separation by LC. Organotin determinations by LC–ICP–MS use mainly ion-exchange chromatography [301,302], ion-pairing [234,280,298–300,313] and reversed-phase [301] liquid chromatography. Ion-exchange chromatography for organotin compounds can be accomplished using a methanol/water (70:30 v/v) eluent containing 60 mM ammonium citrate. However, when applying ICP–MS as a detection device sensitivity reduces significantly due to the high methanol content [302]. Reducing the methanol content of the mobile phase to 30% improved the sensitivity to about 1 ng for TBT, which is still rather

high for environmental analysis. Better results, in terms of sensitivity, can be obtained by ion-pair reversed-phase chromatography [234,298]. However, good separation of alkyltin compounds was only obtained for small alkyl chains [234]: butyltin compounds cannot be detected by this HPLC–ICP–MS approach owing to their higher k' values associated with the molarity of the mobile phase (sodium dodecylsulfate). Increasing the molarity of the mobile phase led to clogging of the sampling orifice of the ICP–MS. Use of ammonium salts overcame this problem. Methyl, butyl and phenyl tins could be separated within 20 min using tris(hydroxymethyl) aminomethane dodecylsulfate as mobile phase and butyl group bonded silicagel as stationary phase [298] Detection limits obtained ranged from 25–50 pg. Reversed-phase ion-pair chromatography (PRP-1 column) using a sodium pentane sulfonate ion pairing agent gives adequate separation and detection limits (1.5–2.6 pg) for methyl, butyl and phenyl tins [300]. The major disadvantage about this chromatographic system is the bleeding of the column after repeated injections of both inorganic and organic tin. Wash-out times of the analytes with the mobile phase was within 30–45 min for organotins whereas 2–3 h were necessary for inorganic tin. Applying an ultrasonic nebulizer improves the nebulization efficiency but even more important is the fact that the desolvation system of the USN removes most of the organic solvent [299]. A fast (6 min) reversed-phase ion-pairing separation with good detection limits in the range of 3–16 pg could be obtained for methyl, butyl and phenyl tins. Again the bleeding of the column was a serious disadvantage of this application limiting possibilities for routine determinations.

Separation by SFC. When coupling SFC to ICP–MS careful consideration must be given to the interface because this interface seriously affects the efficiency of the system. The restrictor must be properly heated (350°C) to prevent analyte condensation and to minimise the Joule-Thompson effect [309]. Due to the low flow rate heated (Ar) make-up gas must be introduced for an efficient sample introduction. A good separation of butyl- and phenyltins were performed with supercritical CO_2 using a capillary column, 10 m long × 50 μm id., coated with a 0.25 μm film thickness of SB-Octyl-50 [173]. Theoretical detection limits (3σ) at an injection volume of 200 nl for tributyltin and tetrabutyltin were 0.025 and 0.035 pg, respectively. However, practical detection limits are worse due to significant peak tailing: <0.06 pg for tetrabutyltin and at 7 pg for tributyltin chloride. In this study no separation of TBT and DBT could be obtained. Another study by this group applying similar stationary phase and column dimensions showed a good separation of tri- and tetra-organotins resulting in detection limits between 0.2 and 0.8 pg [310]. Injection volumes in SFC are low, generally 10–50 nl, therefore practical analysis of environmental significant organometallic compounds seems to be limited.

21.5.5.3 Sample preparation for the determination of organotin compounds by HPLC

In the literature, various methods for sample preparation of aqueous and solid samples (e.g. sediment, soil and biological tissue) for the determination of organotin by HPLC are described [305–308]. In general, after filtration and acidification, aqueous samples can directly be injected in the LC system [306–308]. However, when organotin concentration are expected to be low, a preconcentration step is required. Depending on the type of chromatography applied this can be carried out by extracting the sample with an organic solvent, concentrate it to an appropriate volume and either inject this into the LC or

evaporate the organic solvent completely and re-dissolve it into a polar solvent before injection.

Sediment soil and biological tissues. In solid samples it is necessary to extract the organotin species from the matrix by leaching. First approaches performed an hydrochloric acid leach followed by repeated extraction with an organic solvent and a complexing agent like e.g. tropolone [311]. In this study different types of extraction procedures were evaluated. In terms of chromatographic separation results were poor, mainly caused by co-extraction of organic interferents. Poor separation also affected the accuracy of the method. Good results in this study were obtained by an overnight acetic acid leach. Other approaches involve (ultrasonic) agitation with concentrated acetic acid (in order to extract monobutyltin) and methanol (in order to extract di- and tributyltin) [303–305,307,309]. After centrifuging the solution can be injected in the HPLC-column. Also in this case, when the concentration of organotin is expected to be too low to measure, different methods can be used to concentrate the organotins into a smaller volume. Supercritical fluid extraction of organotin compounds from biological tissue with carbon dioxide is another approach [312]. Although addition of modifiers (e.g. methanol) and complexing agents like dithiocarbamates enhance the extractability from biological tissues, recovery for organotin species from real samples remain poor and are in the range of 30–50% [312].

21.5.6 Determination of lead species by liquid chromatography

21.5.6.1 Introduction

Most applications dealing with the direct determination of organolead compounds in environmental matrices involve a gas chromatographic separation and an appropriate elemental selective detection device. Few applications involving a liquid chromatographic separation have been described in the last decade. Applications for organolead determination with liquid chromatography followed by ICP–AES or AAS detection yield limits of detection in the high nanogram range and are therefore not suitable for environmental analysis. However, ICP–MS detection devices have sufficient selectivity and sensitivity (low pg range) for environmental analysis of organolead species [314–320]. In the literature, the analysis of lead species by liquid chromatography in real samples is limited to aqueous samples like rain water [319], urine [316] and fuel [320]. The following sections will focus on the determination or organolead compounds by ICP–MS detection.

21.5.6.2 Separation and detection of organolead compounds

Separation. Separations of alkyllead compounds by reversed-phase and reversed-phase ion-pairing chromatography have been achieved with reasonable success [315–317]. Due to the high ratio [inorganiclead]/[alkyllead], which is normally observed for environmental samples special care must be taken for the separation of Pb^{2+} and Me_3Pb^+. For reversed-phase chromatography good baseline separations were achieved with 30×3 i.d. mm C_8 column (30 μm particles) as a stationary phase and 0.1 M ammonium acetate buffer (pH 4.7) with 8% m/v methanol as a mobile phase [317]. A reversed-phase ion-pair separation using a 50×1.6 mm i.d. C_{18} column with a mobile phase consisting of 5 mM ammoniumpentane sulfonate in 20:80 (v/v) acetonitrile-water, yields similar results [316].

To sharpen the peaks and reduce separation time a gradient elution can be applied. However, when applying gradient elution for environmental analysis the baseline variations and the blank level are potential problems. When selecting the right gradient it is possible that the baseline is stable or at least linear during the analyte elution and that quantitative results can be obtained [315].

Detection. As mentioned in the introduction of this paragraph ICP–AES and AAS detection devices do not have the required sensitivity. However, sample introduction devices like e.g. a thermospray-microatomizer interface for quartz furnace AAS improve the overall sensitivity for alkylleads to about 1 ng, but remain high for environmental applications [203]. Post column ethylation followed by flame AAS improves the sensitivity again by roughly one order of magnitude [249]. ICP–MS with commercially available spray chambers and nebulizers show a better sensitivity towards the detection of alkyllead compounds (25–100 pg) [314,315] In this last reference the isotope dilution technique is used in the final quantification procedure. More efficient nebulizers like the direct injection nebulizer have the advantage of complete transfer of the analytes to the ICP, resulting in improved absolute detection limits (0.2 pg) [316]. However, relative detection limits remain more or less similar to the normal nebulizer systems due to the necessity of lower flow-rates and subsequent use of microbore columns with low injection volumes. Post column hydride generation of alkyllead compounds in combination with ICP–MS detection can lower detection limits even to the femtogram range (60 fg for trimethyllead) [317]. Finally, alkyllead compounds can be detected by liquid chromatography coupled to laser-enhanced ionization spectrometry (LEIS) [318]. Although absolute detection limits are reasonable (20 pg) practical use remains limited due its high costs.

21.5.7 Determination of mercury species by liquid chromatography

21.5.7.1 Introduction

A good separation of mercury compounds can be achieved by the use of HPLC. During the last decade liquid chromatographic separations of mercury compounds became more popular than GC-based separations. One of the problems occurring in GC-based separation techniques is the interaction of the analytes with metal parts and column material at elevated temperatures leading to, adsorption or decomposition of the analytes and peak deformation (tailing and fronting). Furthermore, GC-based separation needs a derivatization step in order to obtain volatile mercury species. With LC it is also possible to determine less volatile or non-volatile species such as mersalic acid or aromatic organomercurials, which is a problem in GC determinations.

21.5.7.2 Separation of mercury compounds

Mercury species have been mainly separated by ion-pair reversed phase, although ion-exchange chromatography received also some attention [321–334]. In the latter case the separation between ethyl and methyl mercury where disappointing and therefore limited for environmental analysis [321]. In the case of reversed-phase separations 2-mercaptoethanol [322–326,331,332,334] is normally added to the aqueous-organic mobile

phase, although other ion-pairing or complexing agents have been applied, like, e.g., *N,N*-disubstituted dithiocarbamates [323,327–329,333] and L-cysteine [330].

Gradient reversed-phase separations applying a mobile phase of methanol/water (30:70 to 50:50), buffered to pH 5.0 with sodium acetate (0.05 M) and modified by 2-mercaptoethanol (0.1 M) is capable of separating nine organic mercury species (alkyl, arylmercury compounds and compounds with a carboxy group) within 20 min [331,334]. Benzoic mercury is very pH-dependent, increasing retention with decreasing pH [331]. At a mobile phase pH of 5.0 in 30% methanol benzoic mercury is well separated from the other mercury species. Inorganic mercury elutes before the organic mercury species and close to methylmercury. Due to the normally observed high (10^2–10^3) mercury/methylmercury ratios in soils and sediments the separation between these analytes with the above described method is not feasible. A preseparation between organic and inorganic mercury is needed. This can be achieved by a chloroform-water extraction. Acceptable reversed-phase separations can also be obtained by using *N,N*-disubstituted dithiocarbamates as complexing agents. When pyrrolidinedithiocarbamate (PDC) is used as a complexing agent 5 mercury species including inorganic mercury can be separated within 10 min [327]. Methyl, ethyl and inorganic mercury were separated on a reversed-phase column within 6 min. by using 0.5% (m/v) L-cysteine at pH 5 [330].

The use of organic modifiers used in reversed-phase separations are not recommended in combination with the use of standard plasma detection devices. Relatively high percentages ($>30\%$) of organic modifiers in the mobile phases usually leads to higher plasma background, increased instability or even extinction of the plasma. Therefore, generally LC conditions are designed in such a way that as little as possible organic solvents are applied. Low amounts of organic modifiers in the mobile phases (1–3%) can be used without any problems. However, at these percentages of organic modifiers retention of mercury species is quite strong leading to long analysis times.

To overcome these problems alternative mobile phases, such as 5% organic modifier with (0.2 mM) dodecyldimethylammonium bromide vesicles have been suggested [332]. With this type of mobile phases it is possible to separate inorganic from methylmercury within 8 min. Without organic modifiers retention times increase to approx. 17 min. For a fast separation of more than two mercury species this method is not recommended.

21.5.7.3 Detection of mercury compounds

Many spectrometric detectors like, e.g. UV, FAAS, ICP–AES have been applied for liquid chromatography but generally detection limits (nanogram range) are not adequate for direct trace analysis. Sensitivity enhancement can be obtained by a preconcentration step and post column oxidation, see Fig. 21.13 [328]. Good results can be obtained by adding a complexing agent like e.g. PDC to the water sample. The fast formation of a strong and neutral mercury complex can be retained onto a preconcentration column. In this way 300 ml of a water sample was successfully preconcentrated using a 2.5 ml/min flow-rate [328] The mercury dithiocarbamate complexes were desorbed and separated on the reversed-phase column and subsequently decomposed by UV-irradiation in a post-column oxidation step. The next step was a reduction with sodium borohydride resulting in elemental mercury. The mercury vapor was swept from the gas–liquid separator by an

inert gas stream into the AAS. Absolute detection limits obtained were in the range of 0.1 ng. In combination with the preconcentration step this results in environmental significant concentration in the range of 0.5 ng/l.

ICP–MS detection gives a better sensitivity (25–75 pg) for mercury species and is therefore frequently used for mercury speciation analysis [323,324]. When post column oxidation/volatilization is applied absolute detection limits for mercury species as low as 10–20 pg can be obtained [333]. In this case there is no need to have a limited amount of organic modifier in the mobile phase so, optimum separations can be achieved. Recently the use of an in situ nebulizer/vapor generation sample introduction system for ICP–MS was described [330]. In this system the column effluent, without organic modifier, is mixed with an sodium borohydride solution just before introduction into a pneumatic nebulizer. The nebulization process, in which the liquid is shattered into fine droplets in an argon stream, is a very effective way to purge Hg vapor from the liquid. Probably this is more effective than applying a conventional gas-liquid separator in which the Hg vapor is swept by an argon gas. Detection limits obtained for inorganic-, methyl- and ethyl-mercury were in the range of 3–11 pg. The severe problem of clogging the sampler inlet of the ICP–MS by carbon deposition in the case of high concentrations of organic modifiers used for separation can be overcome by adding at least 10% oxygen to the nebulizer gas [333]. In this way the separation can be fully optimized without any limitation for final detection by ICP techniques.

Atomic fluorescence detection is also capable of mercury detection at very low levels and is, like ICP–MS, also frequently applied as a detection device for mercury [326,328, 334]. Normally sample introduction is via the gaseous state after conversion to metallic mercury. In order to enhance the sensitivity water is removed before the argon is swept into the fluorescence cell. This can be performed by chemical drying (e.g. magnesium perchlorate or concentrated sulfuric acid), condensing or selective removal via a Nafion membrane. Detection limits are adequate for environmental analysis and are in the range of 8–20 pg [327,334].

21.5.7.4 Sample preparation for the determination of mercury compounds by HPLC

Water samples. Water samples can be injected into the LC system directly after complexation of the mercury species. If needed an on-line preconcentration step can be introduced to decrease detection limits, as described in the previous section [328].

Sediment soil and biological tissues. Mercury compounds can be extracted with dithizone in chloroform from a slurry of wet sediment/soil with a citrate buffer (pH 2) [325]. After phase separation the mercury-dithizone complex is destroyed with a sodium nitrite solution (color change from green to yellow). The mercury compounds are back-extracted into a sodium thiosulfate solution (1 mM) buffered with ammonium acetate (0.05 mM). In the case of high inorganic mercury concentrations a preseparation of methylmercury and inorganic mercury is recommended because the chromatographic separation can hardly handle large concentration differences between both species. In this case the wet sediment is acidified (6 M HCl) and the mercury species are leached. After centrifugation the supernatant is extracted with toluene to remove the organic mercury species from the inorganic mercury species. Back extraction into a thiosulfate solution is performed in order to introduce the organo mercury compounds into the LC system. A drawback of

TABLE 21.6

MERCURY SPECIATION BY LIQUID CHROMATOGRAPHY

Species[a]	Detector	Chromatography	Comments	Sample	Ref.
1–2	CV-AAS	DDAB modified C_{18} column (10 μm particles), 10 mM ammonium acetate-5% acetonitril-0.005% 2-mercaptoethanol-0.2 mM didodecyl dimethyl-ammonium, 100 μl inj.	NaBH$_4$ reduction (1%) in NaOH (0.1%), DL 1–1.5 ng	Sea water, urine, off-line pre-concentration on 2-mercaptoethanol modified C_{18} (factor 500)	[332]
2	CV-AAS	C_{18} column (3 μm particles), acetonitrile/water (60/40, v/v)-0.5mM PDC at pH 6.5 (acetate buffer)	Post column oxidation (UV)-NaBH$_4$ reduction, DL 40 pg	Destillation as sample digestion, PDC-complexation followed by pre-concentration of complete distilate, (C)RMS for sediment (IAEA 356) and fish tissue (CRM 463-464 and DORM 1)	[329]
1–2	CV-AAS	Cation-exchange, 1 mM acetic acid-1 mM perchlorate-5 mM cysteine at pH 4.4, 25–100 μl inj.	Post column oxidation (UV)-NaBH$_4$ reduction (1 g/l) in 0.1 M NaOH, DL 1–5 ng	Spiked tap water, sample pre-concentration (without complezation) 10–100 ml with micro C_{18} column with counter-flow elution step	[321]
1–6	CV-AAS	C_{18} column (3 μm particles), acetonitrile/water (70:50/50:30, v/v)-0.5 mM PDC at pH 6.5 (acetate buffer), 20 μl inj.	Post column oxidation (UV)-NaBH$_4$ reduction (1.5%) in 1 M NaOH, DL 80–100 pg	Sea water, rain water and process water, Sample pre-concentration (PDC-complexation) 300 ml with micro C_{18} column with counter-flow elution step	[328]

2–9	AFS	C_{18} column (3 μm particles), methanol/water (30:70/50:50, v/v)-0.1 mM 2-mercapto-ethanol-20 mM ammonium acetate, 25 μl inj.	Post column oxidation (S2O82−)-SnCl2 reduction (1%) in 1 M NaOH, DL 20–35 pg	Sediment and biological tissue (DORM-1, LUTS-1), sediment extraction with dithizone-chloroform, back-extraction into thiosulphate solution, tissue digestion first with tetramethyl-ammonium hydroxide further as sediment extraction	[334]
1–3	ICP-MS, insitu nebulizer/vapor (hydride) generator	C_{18} column (5 μm particles), 0.5% (m/v) L-cysteine (pH = 5), 100 μl	$NaBH_4$ reduction (0.1%) in 0.02 M NaOH, DL 3–11 pg	Water samples, RM NRCC NASS-4	[330]
1–4, 10	ICP-MS with HPF/HHP nebulizer	C_{18} column (3 μm particles), acetonitril/water (65/35, v/v)-1 mM PDC at pH 5.5, 25 μl inj.	Post column oxidation (UV), DL 10–20 pg	Distillation as sample digestion, PDC-complexation, pre-concentration of the destillate, (C)RMS for sediment (IAEA 356 and S19) and fish tissue (CRM 463-464 and DORM 1)	[333]

[a] 1, Hg^{II}; 2, CH_3HgCl; 3, C_2H_5HgCl; 4, phenylmercury; 5, methoxyethylmercury; 6, ethoxyethylmercury; 7, nitromersol; 8, tolylmercury; 9, p-mercuribenzoic acid; 10, mersalic acid.

this procedure is the fact that leaching with HCl can result in decomposition of the organic mercury species or incomplete release of the analytes from the sediment/soil [325].

Another approach is adding water, KCl (20% m/v) and H_2SO_4 (8 M) to sediment/soil or biological tissue an perform a distillation (45 min, 180° C with nitrogen) [333]. To the collected distillate a dithiocarbamate complexing agent was added and if necessary preconcentrated or directly injected into the column.

In Table 21.6 some applications for the determination of mercury species are given.

21.5.8 Determination of selenium species by liquid chromatography

21.5.8.1 Introduction

Selenium has been recognized as an essential element for humans, based on its presence at the active sites of glutathione peroxidase. At higher concentrations it becomes toxic for man, animals and marine organisms, depending on its chemical form.

In soil and water, selenium is mainly present as the selenite (Se^{IV}) and selenate (Se^{VI}) ions. Biomethylation of these inorganic species by plants and micro-organisms result in organoselenium compounds. In mammalian tissues the predominant forms of selenium are selenoamino acids like e.g. selenocysteine (SeCys) and selenomethionine (SeMet). Trimethylselenonium (TMSe), as the major metabolite of selenium is mainly determined in urine.

Speciation of inorganic selenium compounds (selenite and selenate) has been extensively studied in natural waters, sediment and soils. However, less attention has been paid to differentiate between the organic species. Most selenium speciation studies distinguish between two main categories of species: (1) non-volatile, which includes inorganic species like selenate and selenite, elemental selenium and matrix bound selenium; and (2) volatile organic selenides like dimethylselenide and dimethyldiselenide. The volatile species are determined with GC while the separation of non-volatile species involve liquid chromatography which will be discussed in the following paragraph.

21.5.8.2 Separation of selenium compounds

Several types of liquid chromatography, including reversed-phase partitioning [335–339], ion pairing [336,340] and ion-exchange [336,341–350] have been applied for the separation of selenium species.

Ion-exchange chromatography is able to separate Se^{IV}, Se^{VI}, selenomethionine and selenocystine when a strong anion exchanger with quaternary functional groups is used with a salicylate buffer at pH 8.5 mobile phase [347,348]. Trimethylselonium cation can been included in these separations, leaving the column unretained, eluting in the dead volume. Separation of six selenium compounds in one run was recently reported [344]. With a 20 mM pyridine solution (pH 5.0) as mobile phase selenic acid, selenous acid, selenocystine, selenoethionine, dimethyl(3-amino-3-carboxy-1-prolpyl)selenonium iodide and trimethylselenonium iodide are separable and quantifiable within 400 s. Selenohomocystine and selenomethionine co-elute under these conditions. Selenomethionine can be quantified under the same chromatographic condition with only the pH adjusted to 2.0. The selenohomocystine concentration can be obtained by the difference between the intensity for the signal selenohomocystine/selenomethionine at pH 5.0 and the intensity of

selenomethionine at pH 2.0. Micro-bore anion-exchange liquid chromatography has also been applied for speciation of selenium compounds [341,343]. Although the presented separation is not improved in comparison with normal bore separations it can be advantageous in the case ICP instrumentation is used. The low effluent flow facilitates direct injection into the plasma resulting in 100% transport efficiency. However, due to the low sample volume to be introduced into microbore LC relative detection limits will not improve compared to normal bore LC in combination with standard nebulizer systems in plasma techniques.

Reversed-phase chromatography, applying a C_{18} bonded silica stationary phase with an ammonium acetate (0.1 M) mobile phase is able to separate three selenium species [339]. Inorganic selenium, selenomethionine and selenoethionine are well separated. Inorganic selenium is not retained due to its ionic character and elutes in the dead volume. In another study a complex mixture of selenium species (selenocystine, methylselenocysteine, selenomethionine, selenoethionine, allylselenocysteine and propylselenocysteine) potentially present in selenium-enriched yeast has been separated within 25 min by applying a reversed-phase C_8 stationary phase with a 98 + 2 water/methanol, 0.1% trifluoroacetic acid mobile phase [337].

A vesicle-mediated reversed-phase LC separation strategy has been applied for the speciation of selenium compounds in urine samples [335]. This LC technique permits the separation of selenocystine, selenomethionine and selenoethionine due to its original C_{18} nature which allows hydrophobic interactions. Moreover, charged compounds, such as selenite and selenate are simultaneously separated as a result of the column modification with didodecyldimethylammonium bromide.

Ion-pair reversed-phase separations have not been described in literature frequently. A combined speciation of four arsenic compounds and selenite and selenate was achieved by applying a PRP1 resin-based reversed-phase column with a 0.5 mM tetrabutylammonium phosphate counterion at pH 8.5 (phosphate buffer) [351].

Parameters like pH, solvent polarity and ionic strength have been optimized for the speciation of three selenium compounds (selenocystine, selenomethionine and trimethylselenonium cation) [340]. Different alkyl sulfonate salts with a variable hydrophobic aliphatic chain (C_5 to C_{20}) were tested. Lauryl sulfonate gave very strong interactions with the trimethylselenonium cation and was therefore discarded. Pentane sulfonate, 0.1 mM in methanol/water (2 + 98) at pH 4.5, gave the best results in terms of retention times and peak resolution.

Three different separation modes were tested in one study for the separation of selenium enriched sample extracts [336]. Ion-exchange, ion-pair and derivatization methods for reversed-phase LC were considered. All techniques have advantages and disadvantages. Anion-exchange chromatography allows the separation of selenite and selenate, but otherwise provides poor separation. Pre-column derivatization and reversed-phase chromatography improves the separation and allows the identification of selenium compounds with terminal amine functionality. Many other selenium species are eluted in the void volume. In this study the ion-pair mode provided the best separation.

21.5.8.3 Detection of selenium compounds

Detection of selenium species by on-line hyphenation of liquid chromatography with

AAS or ICP–AES lacks adequate sensitivity for environmental applications. Absolute detection limits are in the high nanogram range [347]. Again post-column effluent manipulation like, e.g., on-line microwave assisted digestion–hydride generation–QF–AAS or ICP–AES can lower absolute detection limits for selenium species to approximately 0.5–10 ng abs [335,339,345,349]. Atomic fluorescence detection can offer slightly improved (0.2–0.3 ng abs) detection capabilities in comparison to QF–AAS or ICP–AES [350]. The combination of low flow-rates in μ-bore LC with GFAAS exhibits excellent absolute detection limits in the range of 0.04–0.06 ng which is comparable with ICP–MS detection [341]. However, the main disadvantage of this system is the discontinuous character of GFAAS yielding poor chromatography in terms of resolution and long analysis times needed. Furthermore, the low capacity of the μ-bore column with injection volumes of 15 μl, limits the relative detection power of the hyphenation between μ-bore LC and GFAAS. The combination of μ-bore LC with direct injection nebulization plasma techniques has the advantage of a complete sample introduction into the plasma resulting in better absolute detection limits. For ICP–AES detection limits in the range of 0.3–0.6 ng have been reported [343].

However, best detection power is obtained by the hyphenation of liquid chromatography with ICP–MS [335,338–340,346,347]: absolute detection limits are as low as 0.02–0.1 ng abs. Best sensitivities are observed working under high power plasma (1200–1300 W) conditions. Addition of methanol (2–3%) to the mobile phase generally improves detection power by a factor of 2–4. The most abundant selenium isotopes ^{78}Se (23% abundant) and ^{80}Se (50% abundant) suffer from overlap by the ^{38}Ar^{40}Ar and ^{40}Ar^{40}Ar dimers, respectively, and therefore cannot be used in quadrupole ICP–MS. Normally the less abundant ^{82}Se isotope (9% abundant) is used for detection in quadrupole ICP–MS. For selenium speciation analysis there is still a need for lower instrumental detection limits because of the very low natural selenium concentrations in many biological materials. Detection power in ICP–MS could still be improved by applying on-line post column effluent manipulation techniques like have been done in the hyphenation between liquid chromatography with detection systems like QF–AAS and ICP–AES. In theory detection limits could be improved by 1–2 orders of magnitude. Future research will show if detection limits for selenium species as low as 1 pg (abs) are feasible.

21.5.8.4 Sample preparation for the determination of selenium compounds by HPLC

Aqueous samples. Water samples can be injected directly, urine samples after a simple filtration step into the LC system. If needed an on-line preconcentration step can be introduced to improve relative detection limits.

Biological tissues. In a study [343] where different extraction procedures were evaluated it was found that selenium species (Se^{IV}, Se^{VI}, SeMet, SeCys and TMSe) from plant material (CRM 402 white clover) can be best extracted under ultrasonic agitation with a methanol/water (1 + 1) mixture containing 4% ammonia. After a twofold extraction procedure the extracts are combined centrifuged and subsequently filtrated over a 0.45 μm membrane filter. The methanol and ammonia are evaporated and the extract is diluted with Milli-Q water. The extraction efficiency was almost 50% compared with a total selenium determination. The second procedure used also a methanol/water (1 + 1) mixture but now without the ammonia while the third procedure applied 0.3 M hydro-

chloric acid instead of ammonia. These procedures had an extraction efficiency of 29% and 37%, respectively. It was found that extraction procedures using different solvents like aqueous or chloroform extraction or mixtures of water/chloroform/methanol did not yield improved extractability [347]. The maximum water-extraction efficiency reported for the summed selenium species was 15%! Applying enzymatic hydrolysis (pronase/lipase) the extraction efficiency of selenium species from white clover (CRM 402) is slightly worse (42%) then the above described procedure under ultrasonic agitation with a methanol/water (1 + 1) mixture containing 4% ammonia [342]. In the same study this enzymatic hydrolysis was also tested for extraction of selenium species from pig kidney and from yeast. Reported results were 60% and 80%, respectively. Extraction of selenium species from yeast by enzymatic hydrolysis is more effective. Nearly quantitative (> 90%) results were obtained by an enzymatic digestion [337,342].

21.5.9 Determination of antimony species by liquid chromatography

The speciation analysis of antimony has not been reported so frequently as the earlier described elements. Although there are thousands of organic antimony species described in the literature only two inorganic and two organic antimony species are found in the natural environment [352]. The two inorganic forms, Sb^{III} and Sb^V exhibits a higher toxicity than the organic antimony species, methylstibonic acid and dimethylstibinic acid. In ocean waters methylantimony species represents about 10% of the total dissolved antimony with the monomethyl species being predominant. Speciation analysis of these antimony species exhibits much similarity with arsenic speciation analysis. Like in arsenic speciation, antimony speciation can be performed with hydride generation gas chromatography. However, there are some drawbacks in applying the hydride generation technique like: no simultaneously analysis of Sb^{III} and Sb^V, time consuming, and hydride generation is prone to interferences. Therefore a liquid chromatographic separation seems more appropriate. Good separations were reported using an anion-exchange column under alkaline conditions [353]. The direct hyphenation to ICP–MS, operated under high power conditions (>1300 W), provides a technique capable of detecting antimony species around 1 μg/l, which in fact is still a little high to detect background concentrations. For reasons of sensitivity other detectors in liquid chromatography like AAS or ICP–AES are not suitable. Improvement of ICP–MS detection limits, leading to a sensitivity enhancement of a factor of 10–20 were achieved after post-column hydride generation for both inorganic species [354].

Analysis of real soil and natural water samples, high in antimony showed that just one antimony species could be detected, Sb^V [353]. It should be taken into consideration that oxidation of Sb^{III} to Sb^V might have occurred because no special precautions were taken during sampling and transportation. In waste waters (metallurgical industry and municipal discharge) traces of Sb^{III} were found [354]. In this last study the methylated antimony species were not taken into consideration.

21.6 CONCLUSION

One can now consider that the determination of organometallic compounds has been successfully tackled by a wide array of hyphenated systems. If improvements can still be

expected in some of the various ways of hyphenating the different systems between themselves, there is a paramount achievement resulting from the effort of a whole analytical community. Indeed the last 10 years have seen the continuous efforts of the European Community in developing and proposing new certified reference materials for a wide array of species and matrices. Further to the development and certification of these reference materials, every one has learned from their analytical errors during the complex sample preparation, derivatization and detection steps. Procedures for quality control and recovery of the analytes have been fully detailed described, criticized and simplified. This united effort has resulted in the production of valuable CRMs and a good established knowledge [355]. Organometallic species determination in no longer an analytical venture but is now ready to be introduced with confidence in routine analysis.

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Chapter 22

Fundamentals and applications of biosensors for environmental analysis

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CONTENTS

22.1 INTRODUCTION

There is an increased scientific and economic investment for developing production strategies and monitoring procedures to protect the ecosystem and public health from the increasing amount of chemicals released into the environment. These chemicals may undergo biochemical or chemical transformation, leading to new compounds of unknown

toxicity. Both the USA and the EU are introducing new legislation establishing strict limits for the presence of contaminants in air, soil and natural waters.

In order to fulfil these interests and the requirements established by the legislation, it is necessary to have fast and reliable methods of analysis that are economically available for a wide range of private companies and public institutions. Following the flexibility, sensitivity, specificity and efficiency of analyses demonstrated by the numerous immuno-chemical and biological tests now available, research is ongoing to devices combining biodetection with automation, taking advantage of a recent developments in the electronic and microelectronic fields. With this idea arises the concept of *biosensor* as a miniaturized analytical device, comprising on an immobilized biological component (antibody, enzyme, receptor, DNA, cell, etc.) in intimate contact with a transducer (optic, electro-chemical, piezoelectric, etc.) to convert the biorecognition process into a quantifiable electrical signal. This signal can be amplified and subsequently processed to eventually take automatic remedial actions (see Fig. 22.1). The subtle changes in the physicochemical parameters that take place after the biological interaction are thus sensed electronically. A fundamental idea is that the sensor should respond *directly, selectively* and *continuously* to the presence of one or various analytes when in contact with untreated uncollected samples. Consequently the biological reaction should be highly *reversible* to provide *on-site, real* (or *near-real*) *time accurate* measurements.

Nowadays, the term biosensor has been applied to different kind of configurations. Although the above proposed definition corresponds to the operational features of an *ideal biosensor*, in practice most of the devices meet only some of these requisites. For example, most of the immunosensors reported to date do not give a direct answer to the presence of a contaminant but measure a secondary signal product of an enzymatic reac-tion or a fluorescent compound; some devices are not working under fully reversible
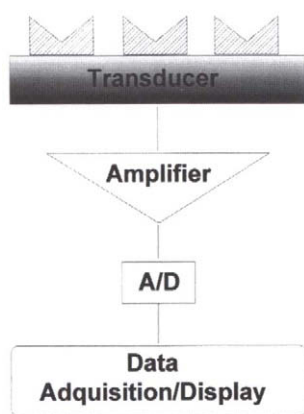
Fig. 22.1. Essential components of a biosensor. The transducer is in intimate contact with a biomolecule that interacts specifically with the analyte present in the sample. Physicochemical changes derived from this interaction are amplified and converted into quantifiable and processable electrical signals. According to the nature of the biological sensing element we can distinguish between two main groups of biosensors: catalytic biosensors and affinity-based biosensors. Repro-duced with permission from *Measurement Science and Technology* (© 1996, IOP Publishing Ltd., UK).

conditions (disposable (or single-use) and reusable sensors) and some others are difficult to bring into a miniaturized size or do not have the appropriate electronic configuration to be used *on-site*. From the operational point of view is interesting to note that although the reported sensors are often included on a flow-through cell, not always work under a continuous manner since the sensing surface should be regenerated before the next measurement takes place. Other sensor configurations appear as probes able to work on continuous or discontinuous way. With a wide idea of what a biosensor can be, throughout this chapter we will try to show the fundamentals and give some examples of the late progress reached by the biosensor technology in the environmental monitoring field.

According to the transducer technology employed biosensors can be classified into *electrochemical, optical, piezoelectric* and *thermometric biosensors*. Another criterion takes into consideration the biorecognition principle contemplating thus *affinity-based* and *catalytic* biosensors (see Fig. 22.1). An appropriate transducer detects the physico-chemical changes associated to the stoichiometric binding event that take place on the *affinity-based* sensors. The different biological sensing elements used as recognition units (antibodies, DNA, protein receptors, cells, tissues, etc.) for environmental monitoring will be discussed below. *Catalytic biosensors* rely on the conversion of a nondetectable substrate into an optically or electrochemically detectable product or vice versa. This process allows the detection of substrates, products, inhibitors and modulators of the catalytic reaction.

22.2 TRANSDUCER TECHNOLOGY

Various transducer systems have been applied for environmental monitoring (for reviews see Refs. [1–7], etc). Electrochemical transducers based on oxidase enzymatic systems dominate most of the environmental applications. However, extensive research is being focussed on optic-based biosensors announcing important advances and realistic applications of these transducers. In this section the basic principles of the major transducers will be presented and their advantages and limitations will be discussed.

22.2.1 Electrochemical transduction

Due to its simplicity electrochemical transduction constitutes a successful route to create low-cost biosensors when coupled to enzymes. However, electrochemical detection of just a biorecognition process is difficult. Catalysis leading to the formation of electroactive substances is frequently necessary. That is the reason because electrochemical immunosensor configurations are often known as electrochemical immunoassays [8–10]. In these immunosensors one of the immunoreactives is immobilized on the transducer and the enzymatic reaction is monitored electrochemically. Electrochemical biosensors are based on amperometric, potentiometric and conductimetric or impedimetric principles.

22.2.1.1 Amperometric principle

This is based on the measurement of the current generated by oxidation or reduction of redox species at the electrode surface, which is maintained at an appropriate electrical potential. The current observed has a linear relationship with the concentration of the
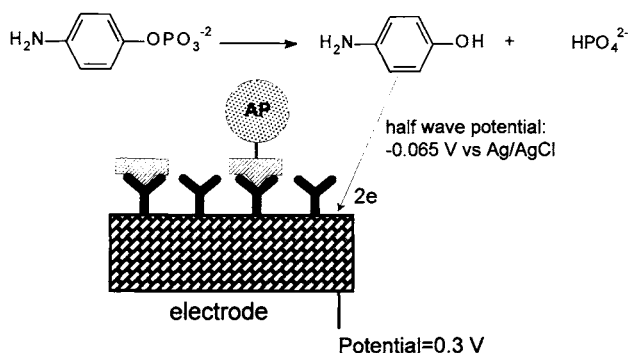
Fig. 22.2. Basic scheme of an amperometric immunosensor. The enzyme employed as label is responsible of the generation of an electroactive product at the potential applied. The current generated can be correlated to the concentration of the analyte in the sample. Reproduced with permission from *Measurement Science and Technology* (© 1996, IOP Publishing Ltd., UK).

electroactive species. The electrode is usually constructed of platinum, gold or carbon. Adjacent to the electrode, entrapped by a membrane or directly immobilized, the enzyme or a receptor are placed. Label enzymes used on the electrochemical immunoassays are usually oxidoreductases such as horseradish peroxidase (HRP), or hydrolytic enzymes, such as alkaline phosphatase (AP), that yield an electroactive specie as a product of the enzymatic reaction (see Fig. 22.2). Other enzymes used as sensing elements for environmental monitoring are tyrosinase, laccase, aldehyde dehydrogenase, etc. Sometimes the substrate or the product of the enzymatic reaction can be monitored amperometrically, without the need of a mediator. These electrodes are called *unmediated amperometric enzyme biosensors*. However, a number of factors must be taken into account when assessing the suitability of an enzyme substrate to be used on an electrochemical detection system: the electrochemistry of the substrate, the electrochemistry of the product of the enzymatic reaction, the medium in which the measurements will be performed and the electrochemistry of endogenous materials in the test sample. A problem often encountered with unmediated sensors is that other species present in the samples being analyzed are also electroactive at the potential applied. For example ascorbic acid and uric acid, present in many biological samples, are oxidized at an anodic potential of $+0.35$ V. AP combined with *p*-aminophenyl phosphate (PAPP) as substrate has been shown to be a good alternative when measuring with such a kind of system. Although PAPP has an irreversible wave in cyclic voltammetry at around 0.45 V vs. Ag/AgCl, its hydrolysis product *p*-aminophenol (PAP) shows a reversible electrochemistry with a half-wave potential of -0.065 V vs. Ag/AgCl. Consequently, measurements on biological matrices can take place at lower potentials avoiding interference of endogenous compounds. Choosing an alternative electron transfer acceptor can also circumvent these problems. Usually the *mediator* is specie of low molecular weight (MW) that shuttles electrons between the redox center of the enzyme and the working electrode. These sensors are called *mediated amperometric enzyme biosensors*. A mediator should react rapidly with the enzyme, exhibit reversible heterogeneous kinetics, possess a low overpotential for regeneration and to be stable at certain range of pH, temperature, redox state and dioxygen. Some

mediators frequently used are I^-, $[Fe(CN)_6]^{4-}$, *o*-phenylenediamine, diaminobenzidine, hydroquinone and 5-aminosalicylic acid.

22.2.1.2 Potentiometric principle

This principle is based on measuring the change in the potential occurred after the specific binding or catalytic process. For affinity-based sensors, direct measurements consider the biomolecules (i.e. proteins) as polyelectrolytes in aqueous solution and consequently their electrical charge is affected by binding the corresponding partner. The potential difference between the working electrode where the biomolecule has been immobilized and a reference electrode is thus measured. A main disadvantage of this principle is that variations in the potential produced by the binding event are too small (1–5 mV). Therefore, the background often limits the reliability and sensitivity of the analysis. In this context, *Silicon field effect sensors* based on the semiconductor technology show better perspectives. A semiconductor is a material characterized by a limited amount of free charge carriers. Impurity doping can, however, increase the total concentration of free charge carriers. Hence, trivalent impurities, such as boron, in a lattice of the tetravalent semiconductor silicon will take up an electron from the vicinity, and the immobile impurity atoms will thus be negatively charged while the surroundings will have an excess of positive mobile charges. The silicon is called to be of the *p*-type. Correspondingly, pentavalent dopant atoms, such as phosphorus, in a lattice of silicon will result in positive immobile ions and negative mobile charges (electrons), and the silicon will be *n*-type. The electrical field applied affects the mobile free charge carriers at the surface of the semiconductor (see Fig. 22.3A,B). This fact makes possible to control the concentration and behavior of the charge carriers by external means. *Semiconductor field effect devices* can be constructed either as *capacitors* or as *field effect transistors* (FET). In a capacitor the change of the capacitance *versus* the voltage applied is recorded whereas in a FET is the current along the semiconductor surface what is measured. Charged or polar species between the gate and the insulator will add their corresponding charge to that of the semiconductor surface and, as a result, the capacitance-voltage curve (capacitor) or the intensity of current vs. voltage curve (FET) will shift. One advantage of these sensors is that miniaturization on a chip and integration into a complete automated system is easily feasible. *Light-addressable potentiometric sensors* (LAPS) are also another application of this principle. LAPS are *n*-type silicon devices possessing an insulating layer in contact with an aqueous solution where the bioreaction takes place. Therefore of the charge distribution at the surface of the insulating layer, a depletion zone appears in the silicon adjacent to the insulator due to electrostatic repulsing forces. The difference with a FET is the mechanism used to detect the changes in the potential at the silicon-insulator interface. LAPS measures an alternating photocurrent (I_p) generated when a light source, such as one of the light-emitting diodes (LEDs), flashes rapidly (see Fig. 22.3C). The photocurrent can only be measured on these discrete zones where the sensor is illuminated. LAPS may thus measure local changes by multiplexing the LEDs and consequently measuring simultaneously different analytes with a single sensor and a set of accompanying electronics. Based on this principle, a device called Threshold™ from Molecular Devices exists on the market (for more information on the fundamentals and potential applications of this kind of sensors see Refs. [11]).
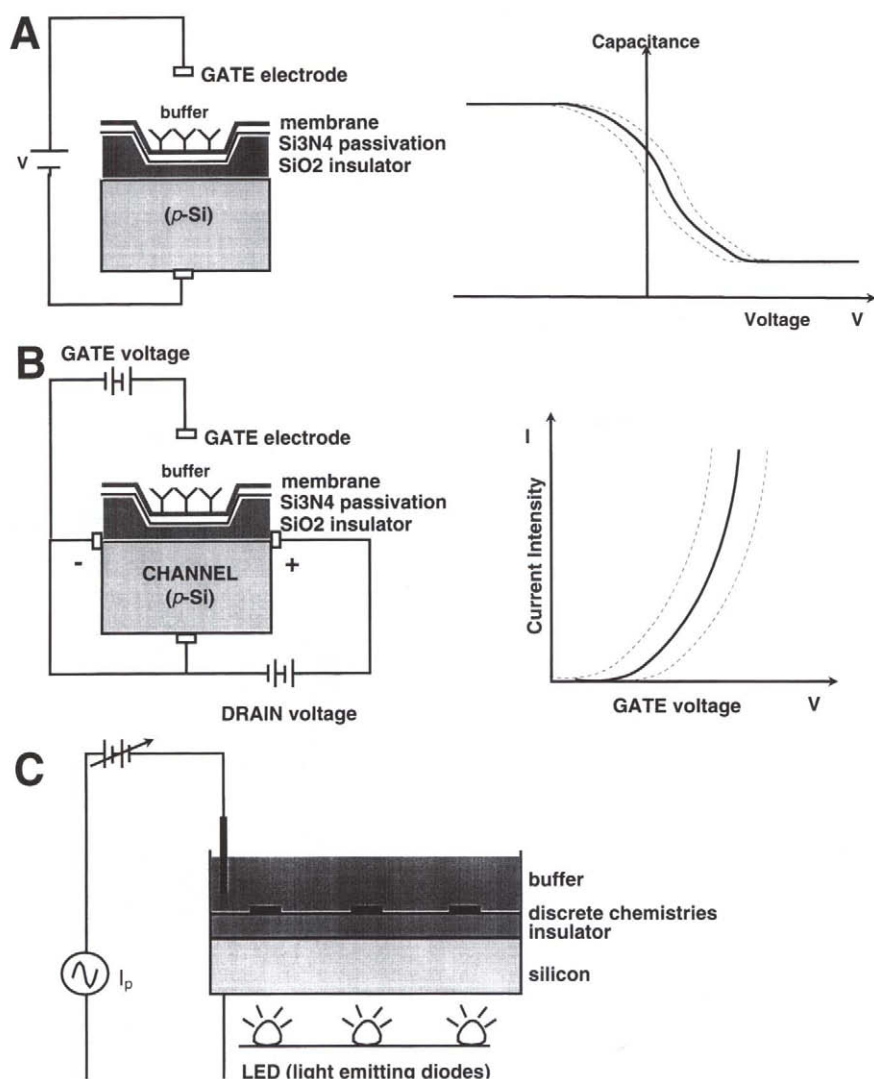
Fig. 22.3.   Basic schemes of silicon field effect potentiometric sensors. (A) Configuration of a capacitor. Capacitance changes as a function of the potential applied. Dotted lines show the shift that can be produced on the curve as a consequence of the antigen-antibody interaction. (B) Schematics of a field effect transistor (FET). Drain current varies with the gate potential. Dotted lines show a parallel shift of the curve cased by the biological interaction. (C) Schematic representation of a light-addressable potentiometric sensor (LAPS). An alternating photocurrent is generated when light-emitting diodes flash rapidly. The accompanying electronics serves to control the potential applied. A and B are reproduced with permission from *Measurement Science and Technology* (© 1996, IOP Publishing Ltd., UK). C is reproduced with permission from *Annual Review of Biophysics and Biomolecular Structure* (vol. 23, © 1994, Annual Reviews Inc.).

22.2.1.3 Conductimetric and impedimetric principle

The physical principle is the same depending on which parameter is measured. Hence, *conductimetry* describes the dependence of the current generated versus a voltage while *impedance* refers to the dependence of the voltage *versus* the current. In both cases a sensing layer placed between a set of two electrodes influences the conductimetric or impedimetric properties.

22.2.2 Optical transduction

First developments of these kinds of sensors took advantage of the flexibility and low cost of the optical fibers measuring the absorption or emission of light of one of the components of the bioreaction. Fiber-optic biosensors usually consist of a fiber-optic strand having an appropriate sensing layer on the distal tip of the fiber. Light travels by the fiber optic by total internal reflection (TIR) until the indicating layer where interacts with one of the components or products formed as a consequence of the bioreaction [12–15]. Changes in the absorbance, luminescence, polarization or refractive index are then detected. In this context, it has also been suggested the possibility of constructing a single device containing an array of individual optical fibers with different selectivities to provide multi-analyte detection. However a few number of analytes can be detected directly regarding their intrinsic fluorescence. Most of the analytes have a characteristic maximum wavelength absorbance; however, the absorbed light is only a very small percentage of the total transmitted. This fact explains why further developments of this area have made use of secondary enzymes or competitive immunoassay configurations using fluorescent labels. On the other hand, extensive research has taken place exploring the effects of the biorecognition step on the optical properties of the media leading to more sophisticated transducing devices. The potential of fiber-optic sensors for environmental analysis has been recently reviewed [16].

22.2.2.1 Evanescent wave (EW) principle

An evanescent wave is produced in the external media (refractive index, n_2) of a waveguide (n_1) by the electromagnetic field associated to the light guided by total internal refection (TIR). The electromagnetic field does not abruptly switches to zero at the interface between the two media ($n_1 > n_2$), but decays exponentially with the distance from the interface (see Fig. 22.4A). The penetration depth of the evanescent field is defined as the distance where is strength is reduced to 1/e of its value at the interface and generally has a value around hundred of nanometers. The penetration depth is dependent of the incidence angle at the interface and is proportional to the wavelength of the excitation light. When molecules with an absorption spectrum including the excitation wavelength are located in the evanescent field, they absorb energy leading to an attenuation (attenuated total reflection, ATN) in the reflected light of the waveguide. One of the advantages of the biosensors based on this principle is that possible interferences from the bulk media are avoided since only directly absorbed substances interfere with the electromagnetic field. However, as mentioned before, the sensitivity reached with this simple setup is often not sufficient to accomplish the EU directives regarding limits of pollutants in the environment (specially

speaking about drinking water, 0.1 μg/ml). For this reason most of the immunosensors reported make use of labeled molecules that are able to re-emit the absorbed evanescent photons at a longer wavelength as fluorescence. Part of this emission is coupled back to the waveguide and in this way is transmitted to the receptor. This phenomenon is known as total internal reflection fluorescence (TIRF). More recently, other EW immunosensor approaches such as *Grating couplers* [17,18] or *Mach–Zehnder interferometers* (MZI) [19–22] have been investigated in order to make possible direct measurement of small analytes without the use of fluorescent labels.

In the grating coupler the change produced in the critical angle, as a consequence of the immunoreaction, is measured. The critical angle is the angle that produces total reflection and is very sensitive to the refractive index and thickness at the sensor surface (see Fig. 22.4B). From this change the N_{eff} (total refractive index due to the effect of the evanescent field) can be calculated. Assuming that n_1 of the waveguide is constant, an optical thickness can be obtained.

On a Mach–Zehnder interferometer the propagating light is splinted in two arms, one of them having the appropriate sensing layer and the other acting as a reference. The evanescent field of the measuring arm collects information regarding the bioreaction, due to the change produced in the refractive index. Consequently the velocity of the wave in this arm varies. At the end recombination of the waves from both arms allows observation of a constructive or destructive interference, which is related to the extent of the bioreaction that has occurred on the sensing arm (see Fig. 22.4C).

For the case of the biosensors based on immunochemical reactions Schipper et al. [22] made a theoretical study of their potential to directly detect environmental contaminants without the need of using competitive immunoassay configurations. They found that the layer growth by a pesticide bound to an immobilized antibody should be of 2×10^{-4} nm, according to the equation $t_f' = \alpha VK[P]$ (t_f' is the maximum average layer growth, $K[P]$ is the fraction of pesticide bound to the antibody, K is the affinity constant and $[P]$ the concentration of the analyte in the sample, V the volume of a single pesticide molecule and α is the maximum available density for pesticide binding sites). Calculations are made estimating that an antibody covers $36\ nm^2$ from where the authors calculate α to be around $2 \times 0.027\ nm^{-2}$. They assume some standard parameters such as an affinity constant (K $10^{-8}\ M^{-1}$), an approximate volume of the analyte ($V\ 0.216 nm^3$ for a pollutant behaving as a cube and having a molecular weight ranging from 200 to 500 Da) and a pesticide concentration ($[P]$) close to the limit established by the EU in drinking water (0.1 μg/l). In the same paper Schipper et al. evaluated *interferometer evanescent wave sensors* such as the Mach–Zehnder immunosensor showing a detection limit of 1×10^{-3} nm layer growth that is quite a close value compared with other direct optic immunosensor setups, although still below the desirable sensitivity. In this context, some authors [19,21] have discussed the necessary improvements to be made in order to reach lower limits of detection on this kind of immunosensor.

22.2.2.2 Surface plasmon resonance (SPR) immunosensors

A surface plasmon resonance is an evanescent electromagnetic field generated at the surface of a metal conductor (usually Ag or Au) when excited by the impact of light of an appropriate wavelength at a particular angle (θ_p). Surface plasmons are generated by
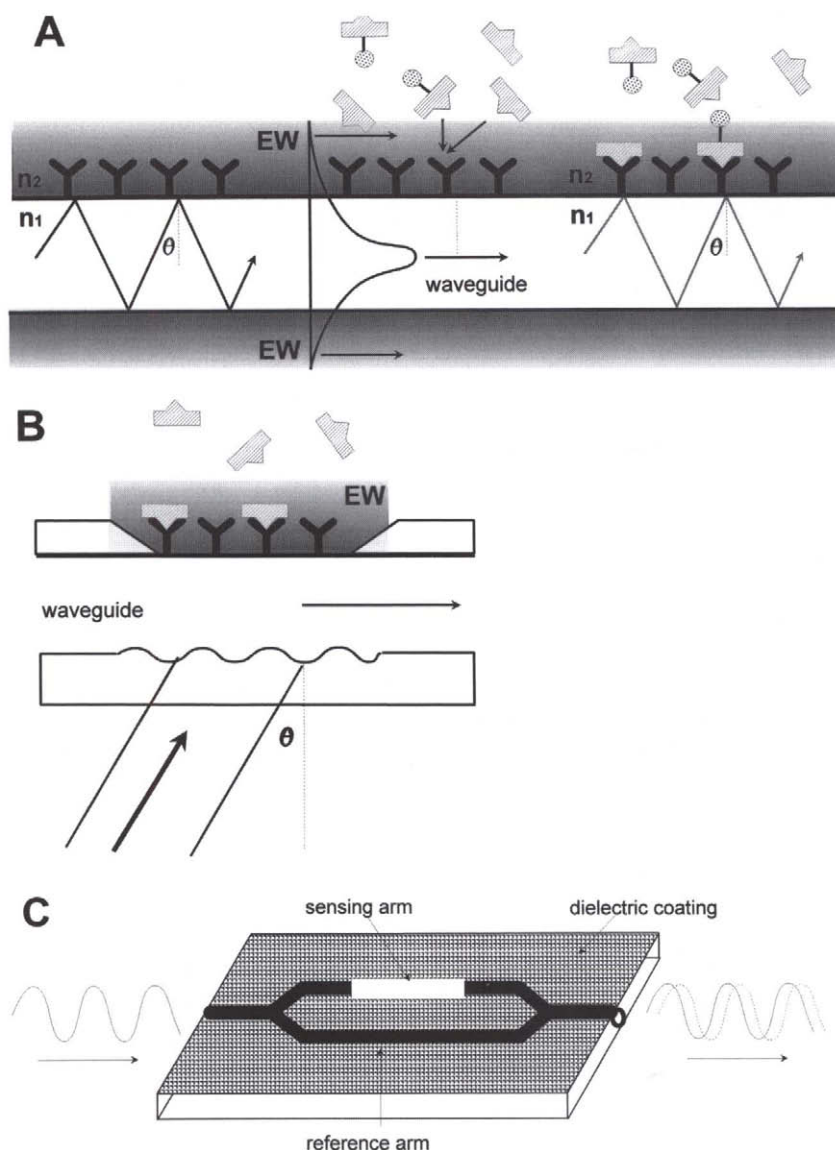
Fig. 22.4. Principles of some optic immunosensor devices. (A) Waveguide evanescent wave (EW) immunosensor. Light directed into the waveguide generates an electromagnetic field that causes direct excitation of a fluorophore used as a label in the competitive immunoassay. The photons are re-emitted and part of this emission is coupled back to the waveguide to the photoreceptor. (B) Grating coupler. The angle causing total reflection is strongly affected by molecules placed on the evanescent field. (C) Mach–Zehnder interferometer. Light is splintered in two arms, one having the appropriate biomolecule and the other acting as a reference. As a consequence of the biological interaction recombination of the waves allows observation of constructive and destructive interferences. Reproduced with permission from *Measurement Science and Technology* (© 1996, IOP Publishing Ltd., UK).

electrons at the metal surfaces that behave differently from those in the bulk of the metal. These electrons are excited by the incident light, producing an oscillation (resonance) at different frequency from that in the bulk of the metal film. The absorption of light energy by the surface plasmons during resonance is observed as a sharp minimum in light reflectance when the varying angle of incidence reaches the critical value. The critical angle depends of the wavelength and polarization state of the incident light, but also of the dielectric properties of the medium adjacent to the metal surface and therefore is affected by analytes binding to that surface (see Fig. 22.5A). This principle allows thus monitoring of biological interactions.

Fisons has also developed a system called IAsys^{TM} to monitor binding events on a real-time mode [23]. IAsys exploits a novel form of optical biosensor that combines the technology of the waveguides with the SPR phenomena. The metal layer is now replaced by a dielectric resonant layer of high refractive index (i.e., titanium, zirconia or hafnia n_1) and separated from the glass prism (n_2) by a low refractive index coupling layer of silica (n_3). The silica layer is thin enough to allow light to couple into the resonant layer via the evanescent field. On the top of this three layers (sandwich configuration of high-low-high n) the immunoreaction takes place. At the interface between the glass and the coupling layer an incident beam of laser light (incident angle is $> \theta$) undergoes TIR. When the resonance angle is reached, a fraction of this light couples into the coupling layer and is directed toward the resonant layer via the evanescent field. At the interface with the sample of lower refractive index (n_4, $n_1 > n_4$), TIR takes place again and sets up a second evanescent wave which interacts with the sample (see Fig. 22.5B). This evanescent wave propagates around 1mm before coupling back to the device. Here a change in the phase of the reflected light is monitored, instead of a variation of the reflected light intensity versus the incident angle. Additionally the propagating light interacts many times with the sample along the waveguide while on SPR this occurs only once. This fact may improve the necessary sensitivity required on environmental analytical applications.

22.2.2.3 Reflectometric interference spectroscopy (RIFS) immunosensors

The basic principle of RIFS immunosensors is the reflected light produced when a light beam passes through a thin film. A light beam passing the interface between two media of different refractive index will be partially reflected. Therefore a thin transparent film will produce an array of reflected beams at each of the interfaces which can be considered as only two reflected beams when the reflectance of the interfaces is small (<0.05). These beams will have a phase difference ($\Delta \Psi$), which is directly related to the physical thickness of the layer. If $\Delta \Psi$ times λ is below the coherence length of the light source the two beams will interfere leading to a modulation of the reflected light intensity as a consequence of the constructive and destructive interference. Changes on the thickness of the film can thus be determined by changes in the interference spectrum [24].

22.2.3 Piezoelectric transducers

Piezoelectrics are materials that may be brought into resonance by the application of an external alternating electric field. The frequency of the resulting oscillation is determined by the mass of the crystal. By coating a piezoelectric with an appropriate biomolecule such
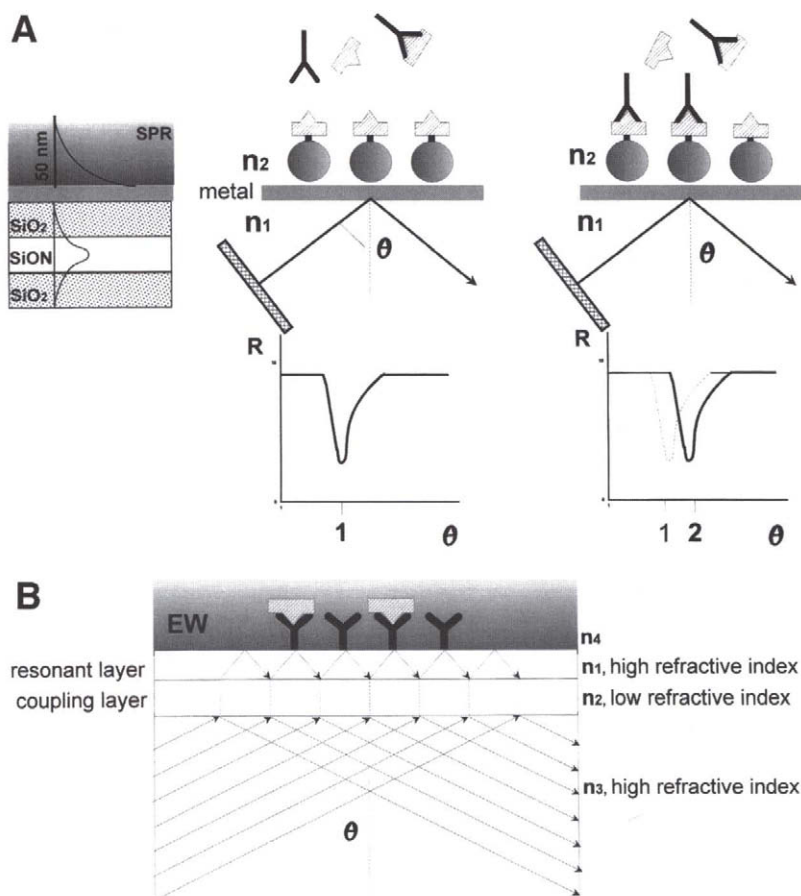
Fig. 22.5. (A) Surface plasmon resonance (SPR). Surface plasmons of a metal conductor are excited by the light energy at a critical angle (θ), causing an oscillation and the generation of an evanescent wave. This condition results in a characteristic decrease of the reflected light intensity. The critical angle is strongly affected by molecules directly attached to the surface of the metal. (B) Resonant mirror (IAsys). At a critical angle, light passes through a coupling layer (n_3) via the evanescent field and couples with a resonant layer (n_1, $n_1 > n_3$) generating a second evanescent field that propagates a certain distance along the surface before coupling back to the device. Reproduced with permission from *Measurement Science and Technology* (© 1996, IOP Publishing Ltd., UK).

as an antibody, these kind of immunosensors can, in principle directly detect the binding of the corresponding analyte (for reviews see [25–27]). Piezoelectric immunosensors may adopt two modes (see Fig. 22.6). (i) *Bulk acoustic (BA) devices* where adsorption of the analytes occurs on the coated surface of a piezoelectric crystal connected to an oscillator circuit. Resonance occurs on the entire mass of the crystal. If for example an antibody-coated crystal is placed in an atmosphere containing the selected analyte the immunoreaction will produce an increase on the mass of the crystal. The resonant frequency will therefore decrease according to the Sauerbrey equation: $\Delta f = -2.3 \times 10^6 f^2 (\Delta m/A)$, where f is the oscillation frequency in Hz, Δm is the adsorbed mass in g, and A is the sensing area

A. BW (bulk wave)

B. SAW (surface acoustic wave)

$$\triangle F = F_2 - F_1 = -2.3 \times 10^{-6} \, F^2 \triangle M/A$$
Sauerbrey equation
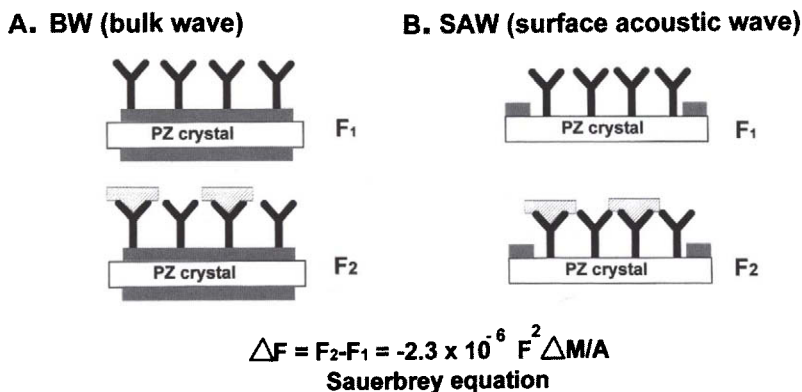
Fig. 22.6. Scheme of piezoelectric immunosensors. The frequency of oscillation of a piezoelectric material is dependent of their mass. (A) On a bulk acoustic device (BA) the entire mass of the crystal will oscillate at a different frequency after the biological interaction. (B) The acoustic wave generated between two sets of electrodes along the surface of the piezoelectric crystal will alter its frequency as a consequence of the immunoreaction.

in cm^2. (ii) *Surface acoustic wave (SAW) devices* where an acoustic wave moves just at the surface of the crystal. Mass loading on the acoustic path between two sets of electrodes will alter the phase wave velocity and cause a shift in the frequency.

This technology can, in principle, detect binding events produced at the surface of the piezoelectric material. Two commercial devices do exist to prepare piezoelectric immunosensors: the PZ 106 Immunobiosensor System (Universal Sensors Inc., New Orleans, LA 70148, USA) and the Model QCA 917 (EG&G, Princeton Applied Research, NJ, USA). The first contains a liquid flow-cell and a computer program to make real time assays of antigen–antibody or receptor–ligand interactions. The second one is designed for simultaneous electrochemical and piezoelectric measurements using a dip or a well holder. Limitations of this technology are the lack of specificity and the interferences produced when used on a liquid media [28–33]. An advantage of the piezoelectric immunosensors is the low cost of the instrumentation required.

22.2.4 Thermistor transducers

Biological processes are generally exothermic and can be detected with a temperature sensor. This concept can be of general applicability independently of the optical or electrochemical properties of the target compound. Additionally, this transducer is virtually free from drift and fouling since it is not really necessary to be immersed in the sample mixture to detect temperature changes. The set-up consists on small reactors where the desired biomolecule is placed usually immobilized on a highly porous support [34]. These columns are attached to thermistor probes connected to a Wheatstone bridge able to reach sensitivities of 100 mV/m°C. Enzymes have been the preferred biorecognition units on this kind of biosensor systems. Thus oxidases offer higher sensitivity than deshydrogenases due to larger reaction heat ($ - \Delta H$, 75–100 kJ/mol). Often the enzymes employed are used in combination with other enzymes in order to increase the enthalpy change. The buffer
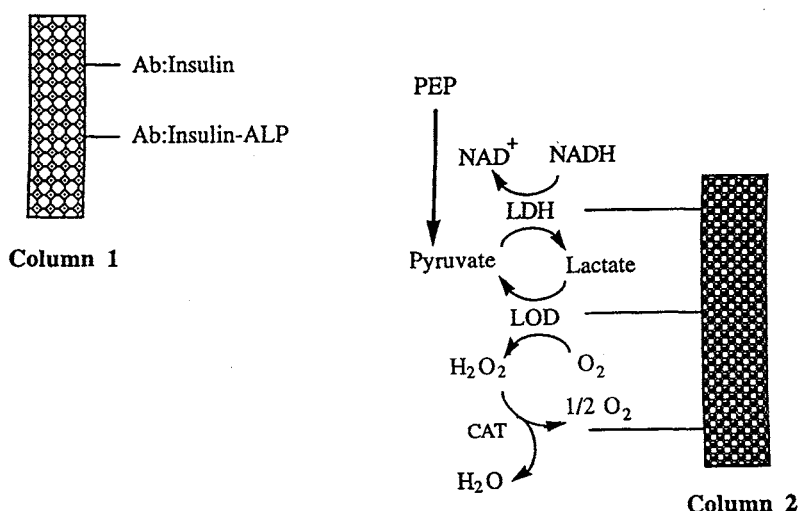
Fig. 22.7. Scheme of a TELISA with substrate recycling detection (from [35]). The first competitive immunological reaction takes place in column A. The pyruvate produced by the alkaline phosphatase (ALP) catalysis of phosphoenolpyruvate (PEP) is transformed again in column B by the action of three other enzymes: lactate dehydrogenase (LDH), lactate oxidase (LOD) and-catalase (CAT). Reproduced with permission from *Frontiers in Biosensorics II. Practical Applications* (© 1997, Birkhaüser Verlag, Basel, Switzerland).

used has also an influence in the final enthalpy variation measured. Thermometric enzyme-linked immunosorbent assays (TELISA) have also been described. The sample is mixed with the enzyme-labeled antigen and introduced in the reactor where the immunosorbent is placed. Usually the recycling of the product of the first enzyme reaction on a second column where other enzymes are immobilized allows an increase in the sensitivity (see Fig. 22.7). Different applications of this principle for on-line biomonitoring processes, clinical and environmental analysis has been reviewed [35]. Current developments for this biosensing principle include devices constructed by micromachining for multisensing purposes and miniaturized instrumentation intended for use in portable monitoring dispositives.

22.3 BIORECOGNITION PRINCIPLE

22.3.1 Affinity-based biosensors

22.3.1.1 Antibodies

The main advantages of these kinds of biosensors are the wide range of affinities available, thus expanding the number of analytes that can selectively detected. The interest of developing biosensing devices based on the use of antibodies to solve environmental problems has growth enormously, as demonstrated on some recent literature reviews [5,6,9,10,27,36–39]. The scope of selectivities of the antibodies is almost unlimited. One proof is the significant amount of antibodies for the detection of trace contaminants (including pesticides, industrial residues and their degradation products) reported on the

last years (see Chapter 7 for antibodies available for different environmental contaminants and for current reviews [40–43]). Additional benefits of using antibodies as sensing elements derive from the possibility to conveniently tailor their affinity and selectivity. Thus, it is known the influence of the hapten design on the final properties of the antibodies (see Chapter 7 and related references [42,44–46]). Immunosensors can also profit from the monoclonal antibody technology which offers a longer supply period of antibodies with defined chemical and biological properties and the chance to carefully screen antibodies having the desired characteristics [47]. Recently, the feasibility of producing recombinant antibodies in hosts other than mouse at lower cost has raised new hopes and opened up several new possibilities [48–55]. Research on the antibody field is still growing and future perspectives also count on the use of small antibody fragments, better defined regarding their chemical structure. This fact would surely help standardization of procedures involved on immunosensor development such as immobilization, stabilization, calibration or storage. It is not the aim of this chapter to enter into the details of obtaining or producing antibodies, although readers are addressed to other several papers dealing with this topic (see Chapter 7 and references therein).

Immunosensors are based on the principles of the solid-phase immunoassays. Physico-chemical events derived from the antibody-analyte recognition phenomenon are extremely subtle and consequently requirements on the transducer are very severe. Environmental contaminants are often small-sized molecules, which complicates even more the detection of the binding event. That is the reason because most of the devices reported to date perform indirect measurements by using competitive immunoassay configurations and/or labels such as enzymes, fluorescent chemicals or electrochemically active substances. Amplification of the signal takes thus place by detecting the physical properties (electroactivity, fluorescence, etc.) of a label or a product of an enzymatic reaction (see Fig. 22.8A) or the binding of the antibody to the sensing surface instead of that of the analyte (see Fig. 22.8B). In order to detect trace level contaminants, indirect measurements are especially necessary if using amperometric and potentiometric electrochemical transducers. However, there have been some practical and theoretical approaches versus direct detection of the analyte using piezoelectric or optical devices (i.e. [21,24,56]).

Another limitation of immunosensors is the fact that antibody–antigen interaction is not readily reversible, in contrast to most of the enzyme-based biosensors where there is a catalytic event. Therefore immunosensors reported to date are generally irreversible single-use or regeneratable devices. Regeneration of the sensing layer takes place by equilibrium displacement of the immunoreaction (i.e. [57,58]), using low affinity antibodies (which may have a direct effect on the sensitivity of the sensor, (i.e. [59]) or by using agents able to disrupt antibody-analyte association such as organic solvents alone or in combination with acidic buffers [60], chaotropic agents [61] or digesting enzymes such as proteinase K [17] (the last procedure is mainly used only when hapten is immobilized at the transducer surface).

A significant number of immunosensor devices have been reported in the literature. Immunosensor configurations based on electrochemical transducers are often known as electrochemical immunoassays. The last advances have been recently reviewed [10] and some examples are given in Table 22.1. The principle is the same but the product of the enzymatic reaction is detected electrochemically. Thus, using a competitive direct ELISA

a) Piezoelectric Immunosensors = mass label (i.e. protein)
b) Optic Immunosensors = fluorescent label (i.e. fluorescein)
c) Electochemical Immunosensors= enzyme catalyzing formation of an electroactive compound (i.e. HRP, AP)
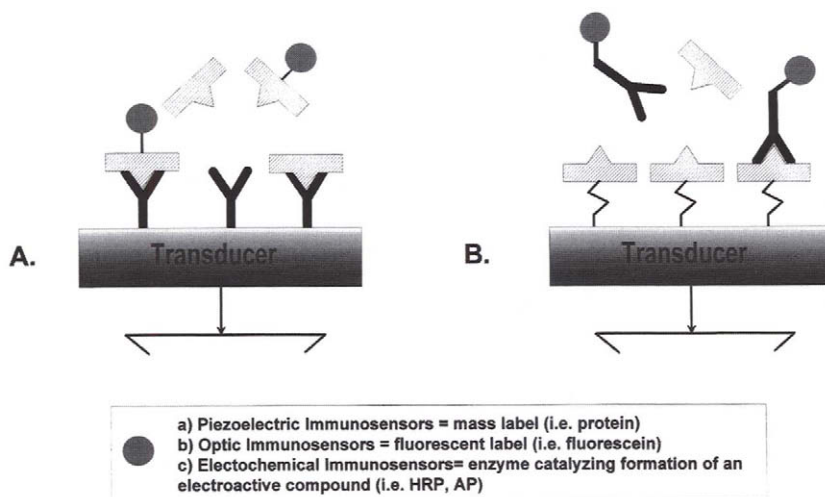
Fig. 22.8. Immunosensor performance is based on the principles of the solid-phase immunoassays. In order to improve sensitivity, immunosensors frequently work under competitive configurations. (A) The analyte competes with a labeled analyte-derivative for the binding sites of the antibody immobilized at the transducer surface. The nature of the label is dependent of the transducer system. (B) The analyte competes with an immobilized analyte derivative for the binding sites of the (labeled or unlabeled) antibody in solution. Binding of the antibody to the sensing surface produces greater physicochemical variations improving the sensitivity of the immunosensor. Reproduced with permission from publishers of *Measurement Science and Technology* (© 1996, IOP Publishing Ltd., UK).

(immobilized antigen), Wilmer et al. [62] have developed an amperometric immunosensor for the detection of 2,4-dichlorophenoxyacetic (2,4-D) acid in water. The enzyme AP catalyzes in the conversion of PAPP to PAP and a limit of detection of 0.1 µg/l can be accomplished. With the same idea, Del Carlo et al. developed disposable screen-printed electrodes for polychlorinated biphenyls (PCBs) based on the use of AP as label on a indirect ELISA format [63]. This enzyme catalyzes the hydrolysis of α-naphthylphosphate to α-naphthol that was detected by different electrochemical approaches. The device is able to reach a detection limit around 0.01 µg/ml.

Immunosensors based in different optical principles have also been reported. In spite of the fact that competitive configurations are often used to reach low limits of detection, the advantage of these transducers is that not always is necessary to use an enzyme label (see Table 22.1, for some examples). Thus, atrazine has been determined using the Mach–Zehnder transducing principle at concentrations around 0.1 µg/l using an indirect competitive assay [64,65]. The binding of the antibody is sufficient to detect significant changes at the trace level. Cyclodiene insecticides can also be detected in the ppb range using an immunosensor based on the EW principle. The antibodies are immobilized on an optic fiber where a competition between the analytes and a fluorescent probe takes place [66]. A portable fiber-optic biosensor based on a fluorescent immunoassay performed on the surface of the optical fiber probe has been applied for the on-site analysis of TNT in groundwater with a limit of detection of 20 µg/l [67–69]. A waveguide surface plasmon

Table 22.1

EXAMPLES OF THE FEATURES OF SOME IMMUNOSENSORS DESCRIBED IN THE LITERATURE FOR ENVIRONMENTAL ANALYSIS[a]

Transducer	Analyte	Competitive	Antibodies M/P	Immobilized reactive	Labeled reactive	Mediator	Detection limit	Ref.
ECl	DNP	Yes	M	Ab	No	No	1nM	[149]
	Phenylacetate	No	M	Ab	No	No	5 μM	[124]
	2,4-D	Yes	P	Ab	Hapten-HRP	5-ASA	40 μg/l	[150]
	2,4-D[b]	Yes	P	Ab	Hapten-HRP	o-Phenylenediamine	1 μg/l	[151]
	2,4-D[c]	Yes	M	Hapten	Ab-HRP	Hydroquinone	0.1 μg/l	[152,153]
	2,4,5-T	Yes	P	Ab	Hapten-HRP	5-ASA	50μg/l	[154]
PZ	Parathion[d]	No	P	Ab	No		35 μg/l	[155]
	Atrazine	No	P	Ab-Protein A	No		0.03 μg/l	[156]
	Atrazine	Yes	M	Hapten	No		0.1 μg/l	[73]
	Atrazine	Yes	P	Ab	Hapten-protein		0.01 μg/l	[75]
	2,4-D[e]e",4>	Yes	M	Hapten	No		1 μg/l	[74]
OP	Terbutryn	Yes	M	Hapten	Ab-F		0.1 μg/l	[157]
	Terbutryn[f g]	Yes	M	Hapten	No		15 nM	[18]
	Atrazine[h]	Yes	M	Ab	Hapten-F		2-5 nM	[158]
	Atrazine	Yes	M	Ab	Hapten-F		0.1 μg/l	[60]
	Atrazine[f g]	Yes	M	Hapten	No		15 nM	[18]
	Atrazine	Yes	M and P	Hapten	No		0.05 μg/l	[159]
	Parathion	Yes	P	Hapten-C	AntiIgG-F		0.3 μg/l	[160]
	Imazethapyr[i",4>]	Yes	P	Ab	Hapten-F		0.3 μg/l	[161,162]
	PCBs	Yes	P	Ab	Hapten-F		10 μg/l	[59]

[a] Abbreviations: EC, electrochemical; PZ, piezoelectric; OP, optical; M, monoclonal; P, polyclonal; 5-ASA, 5-aminosalicylic acid; Ab, catalytic antibodies; DNP, dinitrophenol; PCBs, polychlorinated biphenyls; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid; F, fluorescein; C, casein.

[b] pHFET immunosensor; all the reported devices use potentiometric transducers except c.

[c] Based on amperometric transduction.

[d] Measurement of the concentration of parathion in gas phase; all the measurements are done by the dip and dry method except e.

[e] Measurements are made on a flow-through cell.

[f] Sensitivity value obtained under a kinetic approach.

[g] The transducer is a grating couple.

[h] FOBIA prototype.

[i] Sensitivity value obtained on a dissociation mode. All OP are based on the EW principle except Imazethapyr, which is based on the SPR principle, using a secondary

resonance sensor was used to measure triazine pesticides by coating the gold surface with an analyte derivative and measuring the binding of the antibody on a competitive configuration [70]. The detection limit for simazine was 0.2 µg/l. Analysis carried out on surface and groundwater samples showed a good correlation with the chromatographic system. However, soil water samples resulted in non-selective binding phenomena. The validation of an EW-based prototype developed for monitoring river water samples (RIANA, River ANALyzer) has been validated in our laboratory using real samples collected from the Ebro river area [71,72] (see Table 22.2). Chlorotriazines could be detected, in just 15 min of analysis time, at levels ranging from 0.06 to 0.2 µg/l, depending of the kind of water matrix. The correlation analysis of the results obtained by LC–MS and by the biosensor, regarding total triazine content of the sample showed a regression coefficient of 0.98 and a slope value of 0.73.

In spite of the lack of specificity and the interferences reported when used on a liquid media [28,32,73,74], some applications have been reported on the use of piezoelectric immunosensors for environmental analysis (atrazine [73,75], 2,4-D [74], parathion [155], etc.) Table 22.1 shows the features of some piezoelectric devices used for pesticide determination. It is also worth to note how the greatest sensitivity for atrazine is accomplished when using a labeled (in this case a mass label) immunoreagent [75]. Minunni et al. [73] have studied the performance of a piezoelectric immunosensor for atrazine and found the device to work much better under competitive immunoassay configurations than as a direct device, where interferences due to water adsorption lead to inconsistent results. Recently, the development of a label-free direct piezoelectric immunosensor build on a flow-through cell has been reported [76]. The system was used for the competitive determination of 2,4-D in water in just 25 min with a limit of detection around 0.2 µg/l.

22.3.1.2 Nucleic acids

A general principle for nucleic acid recognition is base pairing leading to the construction of hybridization devices. Such sensors relay on the immobilization of a short (20–40mer) synthetic oligomer or single-stranded DNA probe, (ssDNA probe), whose sequence is complementary to the sought-for target. Exposure of the sensor to the sample containing the target results in the formation of the hybrid on the surface of the transducer. This strategy has been used for detecting a wide variety of microbial and viral pathogens. Electrochemical or optical monitoring of the hybrid have been the most common transducing systems [77,78]. Thus, on electrochemical transduction, the formation of the hybrid is detected by exposing it to a solution with an electroactive indicator that binds the hybrid strongly and reversibly. Although that chemical (urea) or thermal treatments can be use to regenerate the ssDNA probe, single-use biosensors are preferred for on-site environmental analysis. Potentiometric hybridization sensors have been described for detecting important water pathogens such as *Cryptosporidium* (causal agent of diarrheal disease in humans) *Escherichia coli, Giardia, Mycobacterium tuberculosis* [78], etc. The influence of parameters such as the immobilization procedure employed, the kinetics of the hybridization event, the probe length, temperature, and operation potential should be optimized in order to obtain the best conditions. The redox indicators preferred are $Co(phen)_3^{3+}$ or $Co(bpy)_3^{3+}$. Improvement of the detectability is envisaged by integrating these devices with compact microfabricated polymerase chain reaction (PCR) units. Later developments also relay on

TABLE 22.2

LEVELS OF ATRAZINE, SIMAZINE, DEETHYLATRAZINE AND TOTAL TRIAZINES MEASURED IN THE EBRO AREA BY SPE–LC–APCI–MS AND WITH AN EW IMMUNOSENSOR (RIANA)[a]

Sample	Simazine		Atrazine		Deethylatrazine	Total triazines	
	Immunosensor	LC–MS	Immunosensor	LC–MS	LC–MS	Immunosensor[b]	LC–MS[c]
April 1	bdl	0.05	bdl	0.04	0.04	bdl.	0.13
April 2	1.0	0.92	0.46	0.05	n.d.	1.40	0.97
April 3	bdl	0.04	0.16	0.03	0.03	0.10	0.09
May 1	bdl	0.09	bdl	0.09	0.04	bdl	0.22
May 2	0.34	0.37	0.41	0.07	0.03	0.74	0.47
May 3	bdl	0.05	0.37	0.08	0.05	0.40	0.18
June 1	bdl	0.10	bdl	0.07	0.03	bdl	0.20
June 2	bdl	0.10	0.51	0.05	0.04	0.50	0.19
June 3	bdl	0.05	0.33	0.02	0.03	0.30	0.10

[a] Concentrations are expressed in μg/l. Simazine was measured using an anti-simazine antibody, atrazine and total triazines were measured using and anti-atrazine antibody. Sampling points were as follows: (1) Ebre river; (2) Channel; (3) Encanyissada lagoon. LODs were calculated as the signal corresponding to 3 times the standard deviation of the blank signal. bdl, below detection limits.

[b] Total triazine concentration corresponding to the sum of simazine and atrazine.

[c] Total triazine concentration corresponding to the sum of simazine, atrazine and deethylatrazine.

increasing specificity by using probes based on peptide nucleic acids (PNA) which are DNA mimics replacing the sugar–phosphate backbone with a neutral pseudopeptide chain that maintains a proper interbase spacing. These oligomers have probe to be more stable PNA-DNA complexes.

Double-strand DNA probes (dsDNA probes) can also be used as biorecognition molecules of environmental contaminants. Numerous low molecular weight pollutants are carcinogenic or mutagenic compounds based on their ability to bind DNA. Thus, Pandey and Weetall [79] reported an EW biosensor for polyaromatic compounds (7,12-dimethylbenzene, anthracene, 3-methylcholanthrene, etc.), based on their intercalative association with an immobilized dsDNA layer and displacement of a fluorescent marker such as ethidium bromide.

Wang et al. have also developed dsDNA probes for detection of toxic substances, based on electrochemical transducing principles. Thus, electroactive pollutants can directly be determined by intrinsic oxidation of the DNA–pollutant complex at the electrode. Aromatic amines have been analyzed in untreated ground water samples after a short contact period with the modified electrode. It has been reported that a 10-min accumulation period of these amines in the surface of the sensor allows reaching nanomolar detection limits [80]. The observation that the guanine residue can be oxidized at a certain potential has allowed the development of a DNA biosensor for dimethylhydrazine. In the presence of this contaminant N^7-methylguanine is formed and consequently a reduction of the oxidation of this residue is observed on a dose-dependent manner, reaching detection limits below 1 ng/ml [81]. For nonelectroactive analytes it has been suggested the use of competitive and binding displacement of a redox marker from the surface-bound DNA.

The development of DNA-based biosensors is still at a very early stage, although further developments are expected in the future. An interesting aspect of these kind of sensors is that they may offer useful information on the DNA damage capabilities of some pollutants and their degradation products. Additional advantages of this biorecognition principle are the greater stability of the nucleic layers compared with other biomolecules, the feasibility of their synthetic preparation and their potential specificity for screening pathogenic bacteria and viruses.

22.3.1.3 Protein receptors

The use of protein receptors allows environmental pollutant detection based on the knowledge of their mechanism of action. The advantage of this biosensor approach is that families of pollutants interacting specifically with a protein receptor can be detected. In contrast, antibodies are usually addressed to one compound and only few cross-reacting substances are also detected.

Using this concept the photosynthetic reaction center (RC) of the purple bacterium *Rhodobacter sphaeroides* has been isolated and used on different biosensor configurations to detect all herbicides interacting with the photosystem II (PSII herbicides). The RC of these bacteria has a high homology to PSII from plants and therefore may be inhibited by some of the herbicides most frequently used in crops. Biosensor systems using the RC have been described based on different principles (see review [82]). One of them uses a grating coupler optical transducer [83]. A herbicide analog is coupled to the surface of the grating coupler where it competes, after the addition of RC, with the herbicide present in

the sample. Unfortunately the detection limit of this assay is only about 100 μg/l for herbicides such as terbutryn.

A similar biosensor strategy uses as sensing element protein receptors for measuring endocrine disruptors such as xenoestrogens. Receptor binding assays have been described using the same approach as with immunoassays, but using the receptor protein instead of the antibodies [84]. Seifert et al. have been carried out assays using the SPR transducing principle to analyze estrogens and xenoestrogens [85,86]. Other tactics consist in the interaction of the hormone-bound estrogenic receptor with estrogen-responsive DNA or the alteration of the transcription efficiency of the gene containing the estrogen-responsive DNA. Thus, Cheskis et al. developed an SPR-based biosensor that detected changes on estrogen–DNA interaction [87].

22.3.2 Catalytic biosensors

Catalytic biosensors rely on the conversion of a nondetectable substrate into an optically or electrochemically detectable product. This process allows the detection of substrates, products, inhibitors and modulators of the catalytic reaction (see Fig. 22.9). Combination of different enzymes has been described in order to extend the range of detectable analytes by converting a nondetectable primary product to a secondary detectable one by the action of a second or third enzyme [88] or to improve sensitivity by increasing amplification [89].

22.3.2.1 Enzymes

In general, detection of environmental pollutants are either based on their enzymatic transformation or on their capability to act as inhibitors of an enzyme reaction. However, although in nature may exist enzymes able to transform or degrade the pollutant, not
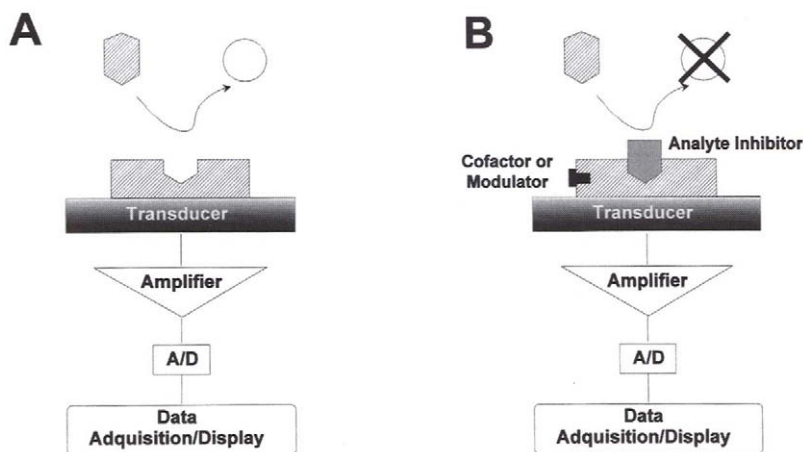
Fig. 22.9. Detection of catalytic biosensors is based on the conversion of a nondeductible substrate into a electrochemically or optically detectable one. This allows direct measurement of the substrate or the product analyte (A) or inhibitors or modulators of the catalytic reaction (B). Reproduced with permission from publishers of *Measurement Science and Technology* (copyright 1996, IOP Publishing Ltd., UK).

always is possible to have them available. Electrochemical transducers are the most frequently used, although there has been also reported the use of fiber optics. The potential of enzyme biosensors on environmental monitoring applications has been reviewed by several authors [88,90–93].

(A) Cholinesterases are by far one of the most employed enzymes to detect environmental contaminants [94–96]. Cholinesterases are commercially available and have a very high stability and sensitivity. Its activity can be measured either using amperometric or potentiometric devices, although devices based on optical [97,98] or piezoelectric [99] transducing principles have also been reported. The activity of cholinesterases is inhibited by a variety of organophosphorus and carbamate insecticides [99–105]. Thus an amperometric-based biosensor has been used for the determination of selected carbamate insecticides (aldicarb, carbaryl, carbofuran, methomyl and propoxur) in vegetable samples and the results compared by liquid chromatography- UV detector [106]. The linear range of the biosensor varied from 5×10^{-5} to 50 mg/kg. The inhibition of an amperometric cholinesterase biosensor by 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid herbicides combined with the use of specific antibodies allowed reaching detectability limits of 10^{-11} M. Similarly, paraoxon and bendiocarb were detected at levels around 10 nM using a LAPS transducer where acetylcholinesterase (AChE) had been immobilized [107]. The advantage of this biosensor is the possibility of simultaneous measurement of eight different samples within minutes.

Some optical-based pesticide AChE biosensors are have also been described. Rogers et al. [108], reported the first fiber-optic anti-cholinesterase biosensor, which was constructed by immobilizing a fluorescein isothiocyanate-tagged acetylcholinesterase (FITC–AChE) on quartz fibers. During acetylcholine hydrolysis, the protons produced quench the pH-dependent fluorescent signal generated by the FITC–AChE present in the evanescent zone. In this way it was possible to detect carbamate insecticides such as bendiocarb and methomyl and organophosphates such as paraoxon in the nanomolar range. Using a different configuration Andres and Narayanaswamy [97] reported a fiber-optic biosensor with an active layer that consisted on AChE immobilized with thymo blue. A reflectance measurement due to the color change produced by the pH during enzymatic reaction allowed limits of detection of 3.1 and 24.7 μg/l for carbofuran and paraoxon, respectively. Microencapsulated cholinesterase in sol–gel crystals has been used to detect organophosphorus pesticides by fluorimetric detection using indoxyl acetate as substrate [98]. Thus pesticides such as naled and mecarbam have been measured in a cuvette and through an optical fiber modified at its end with the immobilized cholinesterase gel in a range of 1.21–11.99 μg/ml and 4.9–328.9 μg/ml, respectively.

A biosensor based on the quartz crystal microbalance has also been reported [99]. Exposure of the immobilized enzyme to a solution of the histological substrate, 3-indolyl acetate, gave rise to the formation of an indigo pigment insoluble product that deposits (precipitates) on the crystal surface. The rate and extent of the enzymatic reaction was followed in real time by measuring the frequency changes associated with the mass changes at the crystal surface induced by the accumulation of the enzymatic reaction product (indigo pigment). The presence of paroxon or carbaryl was then detected by a diminution of the signal (frequency change) arising from their inhibitory effects reaching detection limits of $5.0 \times 10(-8)$ and $1.0 \times 10(-7)$ M, respectively.

(B) Oxidases have been used to directly detect compounds that may act as enzyme

substrates or inhibitors of their activity. The oxidases most frequently used on environmental analysis have been tyrosinases, laccases, peroxidases and deshydrogenases. Thus, for example the enzyme *tyrosinase* catalyzes the orthohydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones while molecular oxygen is reduced to water. This fact has been exploited on amperometric devices to detect contamination by phenolic compounds. Accordingly, immobilized tyrosinase on solid graphite electrodes (SGE) and carbon paste electrodes (CPE) were used as detector units connected to a on-line solid-phase extraction system. The SGE-based biosensor had proven to be useful as a screening device to analyze phenolic compounds in river water samples [109]. With a different approach the capability of various contaminants such as cyanide [110], atrazine [111] diethyldithiocarbamates [112] and hydrazines [113] to inhibit tyrosinase has been reported. In this context Besombes et al. [114] have proposed a tyrosinase-based biosensor as a warming device of accidentally polluted waters. They had found that the amperometric activity of the biosensor was modulated by the presence of several contaminants such as triazines, phenolics, etc. Some of the detection limits reached by this device were 0.4 μM for 3,4-dichlorophenol, 2 μM for chloroisopropylphenylcarbamate (CIPC), 2 μM for 3-choroaniline (the hydrolysis product of CIPC) and 4 μM for atrazine. In this system, using epinephrine as the enzyme substrate, cyanide was detected down to a concentration of 0.02 μM. Similarly, diazinon and dichlorvos were detected at limits around 5 μM and 75 μM, respectively, using a tyrosinase-based oxygen sensor where enzymatic oxygen consumption is monitored electrochemically with the mediator 1,2-naphthoquinone-4-sulfonate [115].

Regarding potential application of biosensors to analyze organic extracts from other types of environmental matrices different from water, some authors have studied the possibilities of using amperometric enzyme electrodes in reversed micellar systems [105,116]. Thus, Liu et al. [116] validated a biosensor for the determination of phenol, 4-chloro-3-methylphenol and 2,4-dimethylphenol. The enzymatic reaction consisted of the oxidation of the phenolic compounds by oxygen, catalyzed by tyrosinase and the reduction of the liberated quinones was amperometrically detected. Similarly, Deng et al. [117] developed an amperometric cryohydrogel tyrosinase biosensor able to work in pure organic solvent; Pita et al. [105] developed an amperometric tyrosinase biosensor for dimethyl- and diethyldithiocarbamates analysis based on inhibition processes in a medium of reversed micelles, reaching limits of detection of 0.074 μM for ziram. General problems encountered with the use of several enzyme biosensors in organic solvent have been reviewed [118].

A solid-state luminescent oxygen sensor has been developed for flow-through measurements using *laccase* as sensing element [119]. The enzyme laccase was immobilized in a minicolumn and used as a recognition system providing specific oxidation of the substrates with the dissolved oxygen being monitored. The enzyme, an oxygen membrane and fiber-optic connector constituted an integral unit, placed on a flow system. The sensor was applied to the determination of polyphenol and other important phenolic compounds in tea, brandy, etc.

Dithiocarbamate fungicides have been measured by their ability to inhibit the enzyme *aldehyde dehydrogenase* (AlDH). As an example, maneb could be detected at concentrations close to 0.05 mg/l [120] by measuring the decrease of the current generated by the oxidation of propionaldehyde to the corresponding carboxylic acid. The detectability of

maneb could be improved to a level of 1.48 μg/l, by using a bienzymatic system based on the combination of AlDH and diaphorase [121]. The NADH formed in the oxidation of propionaldehyde was reoxidized by diaphorase using hexacyanoferrate (III) as electron acceptor. A system sampling atmospheric formaldehyde followed by monitoring the aldehyde using an ion-sensitive field-effect transistor (ISFET) in conjunction with *formaldehyde dehydrogenase* has been reported [122]. The enzyme uses oxidized nicotinamide adenine dinucleoside (NAD) as cofactor catalyzing the oxidation of one mol of formaldehyde and producing two protons, which are sensed by the ISFET. The detection limit reached was about 10 μM for formaldehyde in aqueous solution, which corresponds to an atmospheric concentration in the ppb range. Similarly, a multiple-use biosensor for short-chained alchohols was reported using silicate-encapsulated yeast alcohol dehydrogenase (ADH) and NAD(+) (or ADH/NADH). Changes in fluorescence from the soluble, reduced cofactor upon exposure to alcohols or aldehydes, allows for semiquantitative determination of both substrates in standard aqueous, harsh nonaqueous, and gas-phase environments [123]. Successive exposure to alcohol and aldehyde substrates allows cycling the system, and the gel-matrix provides stabilization and protection of the enzyme in front of hostile and inherently denaturing sample environments, including vapor-phase and nonpolar liquid (e.g., hexane) samples.

(C) Catalytic antibodies may merge the benefits of both immunosensors and catalytic biosensors. They not only bind but also chemically transform the target molecule. To our knowledge only few examples have been reported that exploit the catalytic activity of the antibodies as sensing elements. One of these biosensors uses an antibody able to hydrolyze phenylacetate [124]. The antibody is immobilized on a membrane placed on a pH electrode and the reaction is monitored potentiometrically. In another example, selective binding of Zn(II) to the combining site of an amidase catalytic antibody was suggested as an attractive approach to develop an optical biosensor for this metal [125]. The intrinsic antibody fluorescence was quenched by ligand binding.

22.3.2.2 Whole cells

Although purified single molecules are attractive as sensing elements, their preparation can be expensive. In contrast, whole living cells may be easily isolated from nature (river water, sediments, soil, activated sludge, etc.). Moreover, these whole living cells are less sensitive to inhibition by other compounds present on the matrix, are more tolerant to variations of the pH or of the temperature and seem to have a longer lifetime.

Living cells have been used to assess toxicity or to detect a given group of substances. The first approach is based on the fact that with living cells (or tissues), information on effects over the living systems may be obtained additionally to the analytical aim. When speaking about environmental issues, very often the ultimate objective is the damage produced over the living organisms. In fact, with these biosensors, toxicity effects of even unknown pollutants can be detected. These tests rely on measuring the change of certain biological parameters of the organisms exposed to polluted samples. Toxicity tests have been developed on a variety of taxonomic groups such as invertebrates, fish, plants, algae and microorganisms. This last group has provided the more rapid, direct and sensitive approaches.

Sensors based on the use of microorganisms are often named microbial biosensors and

are usually based on direct or indirect measurement of bioprocesses such as the transformation rate of the carbon, nitrogen or sulfur, enzyme activity, growth, mortality, oxygen consumption or luminescence. Cells with a given type of receptor can be considered as sensors for agonists, although biosensors based on measuring enzyme inhibition have also been described. The sensitivity is determined by the binding constant of the receptor/ligand combination. The reason because bacteria cells have been often used as sensing elements in biosensor field is that they can be genetically engineered to respond to specific substances. This strategy has opened up new specificities and sensor possibilities (see Fig. 22.10). However, specific biosensors have also been reported based on the use of the genes responsible of resistance mechanisms. Microorganisms have evolved a variety of mechanisms that allow them to survive and grow in contaminated environments. The resistance implies the ability of the cell to exclude the toxic by the membrane, sequestration of metals, chemical modification to a less toxic form, etc. The genes responsible of these resistance mechanisms are organized in operons, usually found on plasmids carried by the resistant bacteria. In many cases these plasmids confer resistance to one or more toxic substances. Because of the specificity of these regulation mechanisms, the promoters and
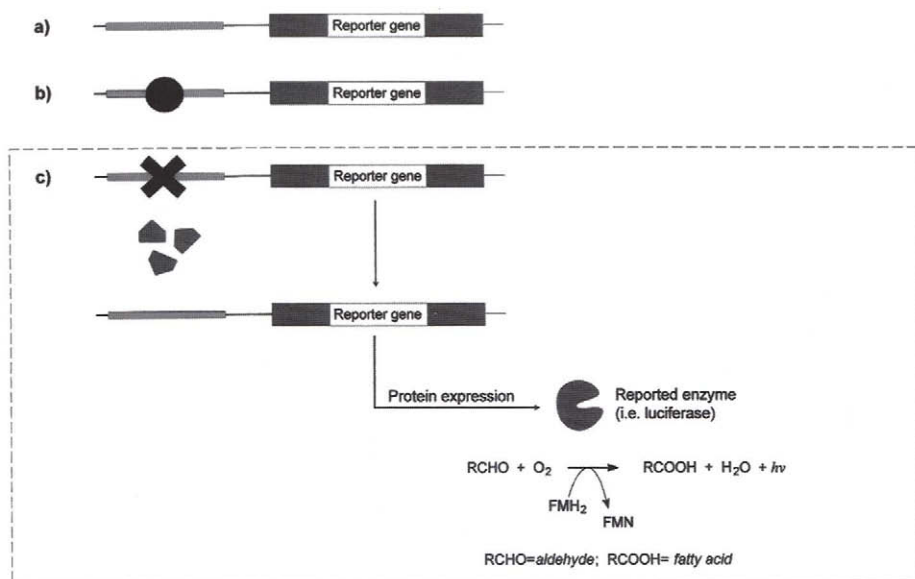
Fig. 22.10. Different sensing principles using bacteria (from [142]). (a) Nonspecific system: the reporter gene is continuously producing the reported protein, but when exposed to toxic substances a reduction of the expression is observed (metabolism inhibition, cell death, etc.). (b) The reporter gene is expressed under the control of a promoter that is responsive to a wide range of conditions (stress, heat-shock, toxic substances, etc.). Exposure to these conditions results in expression of the reporter gene. (c) The promoter is only responsive to a very specific substance or group of substances. The promoter can be positively or negatively regulated by the regulatory protein in the presence of the toxic substance. In the scheme an example is shown of the expression of a protein such as the luciferase enzyme.

regulatory genes can be used to construct promoter-reporter-gene fusion for specific biosensors.

Non-specific biosensors have also been developed based on the heat-shock or stress-response. Exposure to heat, toxic compounds or heavy metals may induce the expression of stress-response genes linked to stress promoters [126]. Induction of bioluminescent proteins or enzymes that can be detected electrochemically using an electrode or a chemi-luminescent substrate allows development of biosensing devices. However, some limitations inherent to microbial sensors are the need of longer response time than enzymes do, and the fact that selectivity is more difficult to accomplish than with single enzymes, due to the variety of metabolic processes occurring on a living cell.

Principles of these kind of biosensors as well as features involved on their construction and applications have been recently reviewed [127–132]. Microbial biosensors have been developed based on different transducing systems. Electrochemical biosensors usually consist of a membrane containing immobilized microorganisms in contact with an electrochemical device. The transducer usually detects variations in redox potential, consumption of oxygen or the appearance or disappearance of an electrochemically active metabolite. Using amperometric microbial sensors it has been possible to detect herbicides [133], benzene [134], triethylamine gas [135], anionic surfactants [136,137], cyanide [138], PCBs [139], phenol [132], chlorinated phenolic compounds [140,141], toxic metals [142] as well as biological oxygen demand BOD) in organic polluted waters [143–145]. The limits of detection accomplished are not always as low as those reached by the antibody-based sensors. Thus, cell respiration and phenol concentration had a linear dependent range between 0.1 and 1 mg/l [132], the cyanide could be measured in the range between 0.3 and 150 μM [138]. An amperometric biosensor has been described for detecting phenols and chlorophenols using *Trichosporon beigelii* (cutaneum). A linear relationship between the current range and the concentration of 4-chlorophenol was observed up to 40 μmol/l and the limit of detection was around 2 μmol/l [146]. A lower limit of detection of 20 mg/l was accomplished by Rawson et al. [133] when detecting herbicides using whole cells with an electron transfer mediator. A biosensor based on linear alkylbenzene sulfonate (LAS) degrading bacteria isolated from activated sludge showed a linear range from 0 to 4 mg/l [136]. Similarly, a biosensor based on the immobilization of *Pseudomonas rathonis* T cells on a Clark-type oxygen electrode provided an analytical system to detect sodium dodecyl sulfate with a lower limit of detection within the range of 0.25–0.75 mg/l [137]. The cells had a plasmid for anionic surfactant degradation. Other detergents such as decylbenzene or alkylbenzene sulfonates only cross-reacted at 36% and 10%, respectively.

Microbial biosensors based on optical transducing systems often rely on the use of bioluminescent bacteria either natural or genetically engineered with a gene for a luciferase that is tied to a promoter sensitive to a given substance. For example, a engineered bioluminescent bacteria *P. fluorescens* HK44 allowed to develop a fiber-optic-based biosensor for naphthalene [147]. The commercially available Microtox® is a nonspecific sensor based on the inhibition of the bioluminescent response of the bacterium *Vibrio fischeri* when exposed to toxic substances such as heavy metals [148]. Similarly, specific microbial biosensors have been described. Thus, the regulatory components of the *mer* operon (gene encoding resistance to mercury in some Gram-positive and Gram-negative bacteria) from *Escherichia coli* have been used for the specific detection of mercury [142].

Similarly, Corbisier et al. [131] constructed by mutagenetic strains of *Alcaligenes eutrophus* able to emit light in the presence of specific heavy metals such as copper ions or families of heavy metals. These strains have been used for the evaluation of incinerator fly ashes and soils contaminated by heavy metals without sample pretreatment.

22.4 CONCLUSIONS

Research on the biosensor field has increased enormously on the last years. Application of such devices to the clinical field is several steps forward than on the environmental area. Field analytical methods may reduce the time and cost of the environmental applications. As field devices, biosensors have advantages to offer over other better established methods such as the immunoassay kits. On the other hand, in performing continuous monitoring a strong competition exists with other implanted and more accepted methods. Although biosensors are not as yet implemented for regulatory purposes in environmental monitoring, they should fulfil the same requirements as conventional robust techniques: (i) acceptable short- and long-term reproducibility; (ii) absence of false negatives above its quantitation limit; and (iii) sufficient robustness when applied to a variety of environmental matrices. In this context, Rogers and Williams from the U.S. Environmental Protection Agency (EPA) have recently discussed several issues that should be considered when facing the development of a new biosensor method [93]. A key question is frequently the limit of quantitation that a biosensor must reach. This requirement is, in fact, dependent of the nature of the matrix. For instance, drinking water regulations are much stricter than that of effluents. The EC establishes very narrow regulations regarding pesticides in drinking water (lower than 0.1 μg/l), whereas the US EPA has set a maximum level fore each pesticide to be measured which is generally based on toxicological considerations. In this respect, the requirements for biosensors will be similar to the analytical techniques currently used. One difference versus conventional techniques, like chromatography, is that biosensors will be tailormade for certain analytes, but not necessarily for all of them.

Future advances on biosensor development will need scientists of different disciplines joining their research efforts in firstly, improving the transducer technology in order to allow direct detection on environmental samples and to confer the necessary flexibility to develop field analytical methods, and secondly, increasing the number of analytes that can be measured using biosensor technology. Especially interesting are those compounds that are difficult to measure using currently accepted methods. Antibody-based biosensors may broaden the number of compounds to be analyzed due to the versatility of the immune system combined with the progress that recombinant DNA technology is accomplishing in this field. Immunosensors can be designed to detect single compounds or a group of compounds. In contrast, enzyme-based biosensors seem better to be addressed to develop class-specific devices, unless they are coupled to chromatographic techniques. Additionally, the possibility exists that other naturally occurring or genetically engineered biomolecules will open the scope of selectivities. On another aspect related to the specificity required, biosensors based on the use of key protein receptors associated to a certain toxic or undesirable effect may offer an attractive and advantageous approach regarding health safety and ecosystem protection, despite the fact that they do not discriminate on the type of chemical. A third trend should be towards developing integrated units able to perform

multianalyte measurements. Finally, the extensive research that is taking place in this area will not reap the expected fruits without performing rigorous validation studies with environmental samples. All these facts will contribute to improve investment by private companies to finally deliver reliable devices into the market.

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